REMARKS

Claims 28-30 and 34-51 are presently pending, upon entry of the amendments herein.

I. The Patent Office Clearly Erred with Respect to Its Handling of Claims 28-30

The Office Action mailed on November 20, 2002 finally rejected claims 34-51 but did not reject claims 28-30. In response, applicant cancelled claims 34-51, without prejudice, for the purposes of advancing prosecution and receiving a formal Notice of Allowance for claims 28-30. Applicant's actions were entirely proper as "All rejections previously made and not continued in the final rejection are considered as withdrawn."[1] In fact, the Federal Circuit in *Paperless Accounting* ruled that the patent application then of concern was no longer rejected under 35 USC § 112 in view of MPEP § 707.07(e)[2] for the reason that the examiner failed to repeat or refer to the previous rejection in the final rejection.[3]

Because claims 28-30 were not rejected in the November 20, 2002 final office action,

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[1] *Waldemar Link, Gmbh & Co. v. Osteonics Corporation, 32 F.3rd 556 at 559 (CAFC, 1994)* (citing *Paperless Accounting, Inc. v. Bay Area Rapid Transit System, 804 F.2d 659, 231 USPQ (BNA) 649 (CAFC, 1986),* quoting *Ex parte Martin, 104 USPQ (BNA) 124, 128 (Supr. Exmr. 1952))*

[2] MPEP 707.07(e): "In taking up an amended case for action the examiner should note in every letter all the requirements outstanding against the case. Every point in the prior action of an examiner which is still applicable must be repeated or referred to, to prevent the implied waiver of the *requirement.*" [emphasis in original]

[3] The Federal Circuit in *Paperless Accounting, Inc.* stated *at 663:*

> " The examiner's rejection of the '196 parent for insufficient disclosure was not "repeated or referred to" in the third or subsequent Office actions. **The MPEP is not permissive in this requirement.** It is notable that only claim 36 was rejected in the third Office action as based on new matter. The '196 disclosure as it then stood, in accordance with MPEP § 707.07(e), was no longer rejected as insufficient.
>
> The district court referred to Halpern's "fail[ure] to prosecute his appeal after the examiner's final rejection of his claims." Had Halpern prosecuted the appeal of the final rejection of claims 39 and 40, as he was entitled to do but did not, the issue of sufficiency of disclosure could not have been raised in such appeal. It was not at issue, because that ground of rejection had not been continued by the examiner. As stated in Ex parte Martin, 104 U.S.P.Q. (BNA) 124, 128 (Supr. Exmr. 1952):
>
> > '**When an examiner fails to mention a rejection in his final action, it has been dropped by the examiner and needs no further response by the applicant.** On appeal, only those grounds of rejection which have been made in the final rejection and commented upon in the examiner's answer to brief are considered by the Board. **All rejections previously made and not continued in the final rejection are c nsidered as withdrawn.** It is not necessary for the examiner to make any specific statement to that effect.' " (emphasis supplied)

the PTO withdrew those grounds of rejection to claims 28-30 in accordance with the Federal Circuit's ruling in *Paperless Accounting*. The claims were thus *de jure* allowed as these claims were no longer rejected under **any** section of the Patent Laws. Accordingly, Applicant cancelled the finally rejected claims 34-51 to place the application in condition for allowance, reserving the right to present claims 34-51 in a continuing application.

Now, the instant Office Action maintains that the rejection of claims 28-30 was omitted in "obvious error." But this is precisely the situation of *Paperless Accounting*; claims 28-30 were – by rule -- allowed and should be so confirmed in a formal Notice of Allowance.

A) Claims 34-51 Must Be Reinstated

Whether or not the Examiner follows the dictates of *Paperless Accounting*, claims 34-51 must be reinstated as the same were cancelled, without prejudice, only in reliance upon the allowed status of claims 28-30. In this regard, Applicant respectfully requests reinstatement of the previously cancelled claims 34-51. Indeed, these reinstated claims – being dependent on allowed claims 28-30 – should be allowed as well.

B) The Finality of the Office Action is Clearly Inappropriate and Should be Withdrawn

The finality of the February 14, 2003 Office Action is clearly inappropriate because the Office Action is now attempting to finally reject claims that have been previously allowed. Under MPEP 706.07(a), second or any subsequent actions on the merits shall not be final where the examiner introduces a new ground of rejection that is not necessitated by applicant's amendment of the claims. As described above, claims 28-30 must be deemed as allowed in view of the November 20, 2002 final office action. Now, the instant Office Action is attempting to

finally reject allowed claims 28-30 under 35 USC 112(1). Applicant respectfully contends that this is a new ground of rejection that is **necessitated by the Patent Office's clear error in handling the claims, and not** by Applicant's amendment of the claims. Accordingly, the finality of the rejection is clearly inappropriate and should be withdrawn under the requirements of MPEP 706.07(a).

C) Even Though All Claims Ought to Receive A Notice of Allowance, the Rejection is Responded To

Although claims 28-30 and 34-51 should receive a formal Notice of Allowance without the need for further submission by Applicant, Applicant presents the following remarks to comply with the requirement under 37 CFR 1.135(c) that Applicant must provide a *bona fide* attempt to fully respond to the outstanding rejections and advance the application to a final action.

II. Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 28-30 inappropriately stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains to make and/or use the invention. Applicant respectfully traverses this rejection because the evidence of record indicates that those skilled in the art in 1998 having Applicant's disclosure available to them would have been able to treat an organism having a disease characterized by the undesired production of a protein as defined by Applicant's claimed invention.

The first paragraph of § 112 requires that the disclosure of a patent application be such that persons skilled in the art, having read the patent application, would be able to practice the

claimed invention. *In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988). There is no legal requirement that this be done in any particular manner. An enabling disclosure can be provided by the use of illustrative examples or simply by broad terminology. *In re Marzocchi*, 169 U.S.P.Q. 367 (C.C.P.A. 1971). The test of enablement is **not** simply whether experimentation would have been necessary, but whether such experimentation would have been **undue**. *See In re Angstadt*, 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *See Wands*, 8 U.S.P.Q.2d at 1404. The factors to be considered in determining whether any necessary experimentation is undue include:

1. the breadth of the claims;
2. the nature of the invention;
3. the state of the prior art;
4. the level of one of ordinary skill;
5. the level of predictability in the art;
6. the amount of direction provided by the inventor;
7. the existence of working examples; and
8. the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

Id. (citing *Ex parte Forman*, 230 U.S.P.Q. 546, 547 (Bd. Pat. App. & Int. 1986)). Any conclusion of non-enablement must be based on the evidence as a whole. *Id.*

When rejecting a claim under the enablement requirement of § 112, the Patent Office bears the "initial burden of setting forth a reasonable explanation as to why [it] believes that the scope of protection provided by [the] claim is not adequately enabled by the description of the invention provided in the specification." *In re Wright*, 27 U.S.P.Q.2d 1510, 1513 (Fed. Cir. 1993). To object to a specification on the grounds that the disclosure is not enabling with respect to the scope of a claim sought to be patented, **the Examiner must provide evidence or technical reasoning substantiating those doubts.** *Id.*; and MPEP § 2164.04. (emphasis supplied) Without a reason to doubt the truth of the statements made in the patent application,

the application must be considered enabling. *In re Wright*, 27 U.S.P.Q.2d at 1513; *In re Marzocchi*, 169 U.S.P.Q. at 369.

Significantly, the Office Action merely doubts the efficacy of using antisense compounds as therapeutic agents, ***in general,*** and fails to provide any evidence or technical reasoning to substantiate these doubts concerning the use of Applicant's oligonucleotides as therapeutic agents ***in particular***. Instead of carrying out an enablement analysis as required by *In re Wright* and MPEP § 2164.04, the Office Action improperly concludes that Applicant's claimed invention is not enabled simply because the field of antisense oligo therapy is unpredictable and/or lacking in enablement – this is impermissibly akin to "guilt by association". Moreover, the Office Action on page 3, second sentence, states that:

> "There is no evidence to indicate how or why the five review papers referenced in the Office action of May 5, 2002, **and which demonstrate Applicants lack of enablement,** mischaracterize the art..." (emphasis supplied)

which, again, inappropriately suggests that Applicant's invention lacks enablement for the simple reason that several review articles have expressed doubts in the field of antisense therapy. The Office Action's improper analysis of the enablement criteria is clearly stated in the sentence bridging pages 4 and 5:

> "...it is reiterated that the primary reasons for lack of enablement of such treatment *in vivo* is due to problems with non-specific interactions of administered oligos with plasma and/or cellular proteins that it encounters before contacting its target, and to significant immune reactions to said administration, and finally to problems with target access itself, that is entering the cell and binding to the transcript so that inhibition occurs".

Applicant respectfully contends that this statement, at best, pertains to several problems noted in the review articles ***in general***, and in no way addresses Applicant's method of using his oligonucleotides as therapeutic agents ***in particular***. In this regard, the Examiner has failed to provide ***specific*** evidence or technical reasoning substantiating any ***particular*** enablement

problems as required under *In re Wright* and MPEP § 2164.04. Thus, the Office Action fails to set forth a *prima facie* case of lack of enablement and the application must be considered as enabled.

The successful *in vivo* administration and treatment of animals with antisense nucleic acids is also well known to the art-skilled. However, the office action inappropriately concludes from a few select references (Branch, A.D., Trends in Biochem. Sci. (1998) 23(2):45-50 ("Branch"); Braasch et al., Biochemistry (2002) April 41(14): 4503-4510 ("Braasch"); Gewirtz et al., Proc. Natl. Acad. Sci., vol 93, pp. 3161-3163 ("Gewirtz"); Agrawal, S. Trends in Biotechnol. 1996, Oct; 14(10):376-87 ("Agrawal"); Tamm, I. et al., The Lancet 2001, Aug. 358: 489-497 ("Tamm")) that the state of antisense-mediated gene inhibition methods *in vivo* for treatment of diseases is highly unpredictable. The Office Action's improper basis for rejection appears to be twofold: (1) the Office Action has inaccurately characterized the state of the art of antisense oligonucleotide chemistry as much more unpredictable than other drug development strategies and (2) the Office Action has improperly required the extreme standard that "undue experimentation means no experimentation required for one skilled in the art" for the application to be enabled.

A. The Office Action Inaccurately Characterizes the State of the Art of Antisense Oligonucleotide Drug Discovery as Much More Unpredictable than Other Drug Development Strategies

Applicant respectfully contends that the state of the art of antisense oligonucleotide drug discovery is just as predictable as that of traditional (i.e., organic chemical compound) drug discovery. As with any new drug, a combination of laboratory and clinical testing is typically carried out to identify and circumvent a variety problems, such as non-specific interactions of administered drugs with plasma and/or cellular proteins that they encounter before contacting its target, and to significant immune reactions to said administration, and finally to problems with

target access itself, that is entering the organism and carrying out its biochemical effect. Indeed, the June 5, 2002 Office Action quotation of Tamm actually suggests the opposite,

> "Tamm et al, concludes by stating that until 'the therapeutic activity of an antisense oligonucleotide is defined by the antisense sequence, and thus to some extent predictable . . . **antisense will not be better than other drug development strategies,** most of which depend on an empirical approach.'" (June 5, 2002 Office Action at 6, emphasis supplied).

Here, the Tamm reference actually is responding to assertions in the antisense field that antisense is actually ***more predictable*** than other drug development strategies. More importantly, it is quite clear from this quotation that **Tamm is clearly not suggesting that antisense is any less predictable than other drug development** using an empirical approach. Indeed, Applicants' claimed methods are plainly stated to be those which treat an organism having a disease characterized by the undesired production of a protein by contacting the organism with "new drugs", wherein the term "new drugs" herein is substituted with Applicant's new oligonucleotides.

Moreover, the Office Action's quotation of the Branch reference for the purpose of showing that the art of using antisense compounds successfully *in vivo* remains highly unpredictable is strained, at best:

> "As is true of all pharmaceuticals, the value of a potential antisense drug can only be judged after its intended clinical use is known, and quantitative information about its dose-response curves and therapeutic index is available." (Feb. 14, 2003 Office Action at 4)

Conversely, Applicant respectfully contends that such statement indicates that the predictability of using antisense compounds successfully **is no worse than all other pharmaceuticals.** In addition, the instant Office Action quotes Braasch and mistakenly alleges that

> "...the preponderance of evidence, as summarized in the review articles cited in the last Office action, indicates that using antisense to provide treatment or prevention of ***any disease is***

> ***controversial and unpredictable***, as indicated by Braasch..."
> (Office Action at 3, emphasis supplied).

This statement is clearly false in view of the clinical studies of multiple antisense drugs that were underway prior to the publication of the cited review articles. Indeed one of these studies resulted in FDA marketing approval of an antisense drug. Accordingly, the Office Action has mischaracterized the state of the art by pointing to a select few references that make overbroad and erroneous statements regarding certain aspects of antisense oligonucleotide drug development. In fact, the references' respective statements have proven to be wrong, thus leaving the Examiner's allegations totally unsupported.

The Office Action mischaracterizes Braasch, for example, for the proposition that the state of the art of antisense oligonucleotides is unpredictable and that the technology, in general, is unreliable. Braasch, however, actually cuts against the Office Action's position regarding the state of the art at the time of Applicants' effective filing date. Specifically, the Office Action cites to the first full paragraph of Braasch, in which it states that "over the past decade ... gene inhibition by antisense oligomers has not proven to be a robust or generally reliable technology . . ." This passage merely notes ***past*** problems (*i.e.*, those known before Braasch) that since have been solved. Indeed, at page 4504, Braasch teaches (referring to references 8 and 14-16) that those skilled in the art know how to deliver antisense oligonucleotides into various organs of both animals and humans (*see,* Braasch at 4504 under the heading "Uptake by Cultured Cells and Tissue Distribution"). Accordingly, Braasch actually supports Applicants' position that the instant claims are enabled.

The Office Action's reliance on Branch is also flawed because clinical use data, quantitative dose-response curves and therapeutic index are not required to be demonstrated by the first paragraph of 35 U.S.C. § 112. Enablement does ***not*** require that the claimed invention satisfy the higher safety standards applied to drugs to be used in clinical trials. According to

MPEP § 2107.03, "Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials .... it is improper for office personnel to request evidence...regarding the degree of effectiveness [in humans]." (emphasis in the original) Enablement requires only that the application teach how to make and use the invention without undue experimentation. This requirement has been met: one having ordinary skill in the art would be able to make and use the inventions without undue experimentation using only the application as a guide.

In regards to the concerns voiced by Branch and repeated in the Office Action that "the value of a potential antisense drug can only be judged after its intended clinical use is known...", it turns out that antisense drugs were, in fact, already being tested *in vivo* in a clinical setting like other experimental pharmaceuticals. For example, the oligonucleotide antisense drug Fomivirsen (ISIS Pharmaceuticals, Inc. ("ISIS"), the assignee of the instant application) was approved for treatment of cytomegaloviral-induced retinitis by the FDA in 1998. More importantly, the Investigational New Drug Application ("IND") for Fomivirsen was filed with the FDA in 1993 – five years ***prior*** to Branch's publication. Thus, unbeknownst to Branch, while he was questioning the use of antisense oligonucleotides *in vivo*, those skilled in the art were already gathering such data *in vivo* in support of their IND well before 1993. Many other oligonucleotide antisense drugs are currently involved in clinical trials (*see, e.g.*, Tamm et al. (the Lancet (2001) 358:489-497 at 490)).

Significantly, a search of the art of antisense oligonucleotides reveals approximately 16,986 references that comprise the "state of the art" of antisense oligonucleotide chemistry (*see* Exhibit A).[4] Out of the 16,986 references, the instant Office Action has focused on select statements in ***five*** references to characterize the entire state of the art. While surveying the

4 The entire search spanned a total of 850 pages. Selected pages of the search are included as Exhibit A.

teachings of each of the 16,986 references is not practicable here, Applicant submits that, not only has the Office Action not considered the art that contradicts the statements in the five references selected upon which it relied, but that some of the statements relied upon by the Office Action have been clinically proven to be ***false***.

In addition to Branch, Braasch and Tamm, the earlier June 5, 2002 Office Action also inappropriately relies on Gewirtz and Agrawal to show alleged unpredictability in the art, alleged lack of stability, specificity, and cellular uptake of oligonucleotides, as well as alleged lack of accessibility of binding sites. Significantly, however, it is improper to base a conclusion about the entire state of the art of antisense oligonucleotide chemistry upon these few references when so many other references contradict their teachings.

For example, attached hereto as Exhibits B-D are selected chapters form a book entitled *Antisense Drug Technology* (Marcel Dekker, Inc., (2001)). This book is a compilation of antisense-related references published throughout the 1990's, which, for the most part predate Applicant's earliest priority date. Chapter 5, entitled "Methods of Selecting Sites in RNA for Antisense Targeting" (Exhibit B), and the supporting references cited therein, illustrate that those skilled in the art do indeed consider antisense oligonucleotide chemistry to be predictable. For example, Chapter 5, at page 111, states that "[o]ne attraction of antisense technology is that high specificity can be achieved." The supporting references that are citied in support of this statement, *i.e.*, references 11, 63, and 64, were published from 1994 to 1999. Accordingly, Chapter 5 of Antisense Drug Technology, along with the supporting references cited therein, teaches that the state of the art of antisense oligonucleotide chemistry is indeed predictable and that those skilled in the art would not have had to partake in undue experimentation to achieve binding specificity, thus directly contradicting the allegations of undue experimentation in the June 5, 2002 Office Action on page 7.

Likewise, Chapter 7 of Antisense Drug Technology entitled "Suborgan Pharmacokinetics" and the references listed therein (Exhibit C) teach that the efficacy and safety of certain oligonucleotides in various animal models and in the clinical setting have been well documented (page 111). Chapter 7 further teaches that, in various animal models, not only can oligonucleotides be given directly without a carrier, but that such oligonucleotides are consistently (*i.e.*, ***predictably***) and unequivocally localized within the cells of various organs. Chapter 8 summarizes the art with respect to modulating the activity or production of proteins with respect to human subjects (Exhibit D). Accordingly, Chapters 6 (Exhibit I) and 8 of Antisense Drug Technology, along with the supporting references cited therein, teach that the state of the art of antisense oligonucleotide chemistry is indeed predictable and that those skilled in the art do not have to partake in undue experimentation to modulate the production or activity of a protein in an organism, thus directly contradicting that alleged in the Office Action.

Another reference that was not considered by the Office Action is Whitesell et al., Antisense Res. Dev. (1991) 1:343-50 ("Whitesell") (Exhibit E). Whitesell teaches that those skilled in the art as far back as 1991 were successful in *in vivo* modulation of N-myc expression in mice. Similarly, Mirabelli et al., Anti-Cancer Drug Des. (1991), 6, 647-661 ("Mirabelli") (Exhibit F) evidences the state of the art as far back as 1991. At page 651, fourth full paragraph, Mirabelli notes that "the therapeutic indexes of phosphorothioate oligonucleotides appear to be quite high" and that "certain phosphorothioates . . . are extremely well tolerated in animals." Accordingly, Whitesell and Mirabelli provide evidence that the state of the art of antisense oligonucleotide chemistry was not as embryonic as the Office Action would lead one to believe. Moreover, Whitesell and Mirabelli provide evidence that as far back as 1991, one skilled in the art would not have to conduct undue experimentation to modulate the production

or activity of a protein in an organism, such as for treating a disease characterized by the undesired production of such a protein.

Yet another reference not considered by the Office Action is Crook, Ann. Rev. Pharmacol. Toxicol. 1992, 32:329-76 ("Crooke") (Exhibit G). Crooke is a 1992 review of the art and, at page 342, provides evidence that those skilled in the art were conducting *in vivo* pharmacokinetic data in mice and rats.

Still another reference not considered by the Office Action is Cossum et al., The Journal of Pharmacology and Experimental Therapeutics (1993) 267:1181-1190 ("Cossum") (Exhibit H). Cossum discusses non-antisense effects and avoiding them *in vivo* by not using doses that are significantly greater than the antisense effective dose. Thus, by 1993, the skilled artisan knew how to avoid non-specific effects. Moreover, Cossum evidences that, by 1993, those skilled in the art were delivering antisense oligonucleotides *in vivo* where the only carrier was a phosphate buffer.

The Office Action has clearly ignored the state of the art as a whole. Indeed, as indicated above, the "state of the art" of antisense oligonucleotide chemistry includes much more than the five references upon which the Office Action relies. Moreover, as evidenced by the drug Fomivirsen and other compounds that are in clinical trials, those skilled in the art have proven that much of the prophetic criticism cited in the Office Action was simply wrong. Accordingly, the Office Action has unfairly mischaracterized the state of the art of antisense oligonucleotides. For this reason alone, the rejections for alleged lack of enablement should be withdrawn.

B. The Office Action Improperly Requires an Extreme Standard that *"Undue Experimentation Means No Experimentation Required for One Skilled in the Art"* for the Application to be Enabled.

Applicant's specification includes many examples demonstrating the use of his new oligonucleotides in both *in vitro* and in *in vivo* studies. The *in vitro* studies show the modulation of ICAM-1 protein expression in HUVEC cells, while the *in vivo* studies show the modulation of protein binding and tissue distribution in male Sprague-Dewey rats using I.V. bolus administration of ^{3}H radio labeled oligonucleotides. Applicant respectfully contends that one skilled in the art, equipped with his specification, would be able to treat organisms having a disease characterized by the undesired production of a protein, such as ICAM-1, with his new oligonucleotides. However, in rejecting Applicant's claims for alleged lack of enablement, the Office Action has improperly required Applicant to provide more than the supplied combination of *in vitro* and *in vivo* studies to prove enablement (Office Action at 5) – the Office Action seems to require actual clinical data showing protein expression was indeed modulated in an organism. Thus, the Office Action expresses the position that Applicant not only should carry out *in vivo* experiments to prove enablement (as Applicant as done), but such *in vivo* experiments should preferably report a variety of data, which pertain to problems associated with:

> "non-specific interactions of administered oligos with plasma and/or cellular proteins that [oligos] encounter before contacting [their] target, and
>
> to significant immune reactions to said administration, and finally
>
> to problems with target access itself, that is entering the cell and binding to the transcript so that inhibition occurs."
> (Office Action at 5, parsing supplied)

Applicant respectfully contends that such data is comparable in breadth to that required by the FDA and generated in clinical trials, which is clearly not required to show enablement ***of any***

new drug treatment under the patent laws. The Office Action's calling for this quantity of data amounts to requiring the completion of clinical studies and ascertaining the molecular biological mechanism between the oligo and its target – demonstrating that the Applicant had this amount of data is clearly not required by the first paragraph of 35 U.S.C. § 112. Indeed, enablement does ***not*** require that the claimed invention satisfy the higher safety standards applied to drugs to be used in clinical trials. According to MPEP § 2107.03,

> "Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials...it is improper for office personnel to request evidence...regarding the degree of effectiveness [in humans]." (emphasis in the original)

Enablement requires only that the application teach how to make and use the invention without undue experimentation. This requirement has been met -- one having ordinary skill in the art would be able to make and use the inventions without undue experimentation using only the application as a guide. In view of the synopsis of the state of the art of antisense oligonucleotide chemistry provided above, Applicants' claims are nonetheless enabled with respect to treating organisms characterized by the undesired production of a protein.

As detailed above, the art as described in the chapters from the collection entitled "Antisense Drug Technology" (Exhibits B-D), Whitesell (Exhibit E), Mirabelli (Exhibit F), Crooke (Exhibit G), Cossum (Exhibit H) and even those references relied upon in the Office Action such as Braasch, Branch and Taam, show that those skilled in the art indeed knew how to modulate the activity or production of a protein for treating an organism with at least some measurable amount of success.

The Office Action unfairly (and incorrectly) minimizes the well-documented evidence that those skilled in the art would know how to use the oligonucleotides, as claimed by the Applicant, to treat a disease *in vivo*. As noted above, the antisense oligonucleotide drug

Fomivirsen was approved for treatment of cytomegaloviral-induced retinitis by the FDA in 1998, and its Investigational New Drug Application ("IND") was filed with the FDA in 1993. Significantly, pre-clinical animal experiments were performed and completed prior to filing the IND for Fomivirsen in 1993.

There is no requirement under the first paragraph of 35 USC § 112 that Applicant must actually treat an organism having a disease characterized by the undesired production of a protein. Indeed, all that is required is that those skilled in the art be able to use the invention to some measurable extent. Since this requirement has been satisfied (as is well-documented in the art), Applicants respectfully request that the rejection for alleged lack of enablement be reconsidered and withdrawn.

Conclusions:

Applicant requests the Examiner to:

(1) reinstate claims 34-51;

(2) withdraw the finality of the rejection;

(3) reconsider and withdraw the rejection of the claims; and

(4) pass claims 28-30 and 34-51 to allowance.

If the Examiner is of contrary view, the Examiner is requested to contact the undersigned attorneys at 215-568-3100.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE**".

Attached hereto are **Exhibits A-I** as described herein.

Respectfully submitted:

Date: March 25, 2003

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the claims:

Please reinstate the following claims:

34. The method of claim 28 wherein R_1 is -O-CH_2-CH_2-O-CH_3.

35. The method of claim 28 wherein n is about 5 to about 50.

36. The method of claim 28 wherein n is about 8 to about 30.

37. The method of claim 28 wherein n is about 4 to about 15.

38. The method of claim 28 wherein n is 2 to about 10.

39. The method of claim 29 wherein R_1 is -O-CH_2-CH_2-O-CH_3.

40. The method of claim 29 wherein R_2 is H, and R_3 is OH.

41. The method of claim 29 wherein R_2 is a phosphodiester-linked oligonucleotide or a phosphorothioate linked oligonucleotide.

42. The method of claim 29 wherein R_3 is a phosphodiester-linked oligonucleotide or a phosphorothioate linked oligonucleotide.

43. The method of claim 29 R_2 and R_3 are each a phosphodiester-linked oligonucleotide or a phosphorothioate linked oligonucleotide.

44. The method of claim 30 wherein R_1 is -O-CH_2-CH_2-O-CH_3.

45. The method of claim 30 wherein R_2 is H, and R_3 is OH.

46. The method of claim 30 wherein n is about 5 to about 50.

47. The method of claim 30 wherein n is about 8 to about 30.

48. The method of claim 30 wherein n is about 4 to about 15.

49. The method of claim 30 wherein n is 2 to about 10.

50. The method of claim 30 wherein W^2 is a plurality of covalently bound nucleosides linked by phosphodiester linkages.

51. The method of claim 30 wherein W^2 is a plurality of covalently bound nucleosides linked by phosphorothioate linkages.

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5

Methods of Selecting Sites in RNA for Antisense Targeting

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I. INTRODUCTION

The mechanism of action for antisense oligonucleotides requires that the oligonucleotide hybridize to its mRNA target. Therefore, in principle, design of an antisense oligonucleotide requires simply that the oligonucleotide be complementary to the mRNA. In practice, however, when several oligonucleotides complementary to an mRNA are screened, certain antisense oligonucleotides are more active and more potent than others in suppressing specific gene expression (1–12). In addition, some complementary oligonucleotides can show nonantisense effects (13–15). The most commonly used and most effective approach to discovery of antisense oligonucleotides involves synthesis of numerous oligonucleotides (up to several dozen) designed to hybridize to different regions of the targeted mRNA, followed by activity screening in cells (16).

Several attempts have been made to identify features of oligonucleotides that are associated with antisense activity. Development of successful methods for selection of active oligonucleotides prior to oligonucleotide synthesis and cell-based screening would have two benefits. First, the cost of antisense discovery would be reduced. If a computer algorithm could pick the most active compound for an antisense target, then synthesis and screening of multiple compounds could be eliminated. Second, identification of the features associated with specific and nonspecific effects of oligonucleotides would likely lead to a better understanding of the detailed mechanism of antisense activity and, potentially, to identification of compounds with even greater potency. This review will discuss methods that have been used to select antisense oligonucleotides, the

effectiveness of these methods, and the prospect for improved methods in the future.

II. RNA STRUCTURE CALCULATIONS

It has long been assumed that activity of an antisense oligonucleotide is directly related to the hybridization affinity of the oligonucleotide for its mRNA target. Support for this assumption comes from the observation that, at a given target site, longer oligonucleotides are more active than shorter ones (17). In addition, at a given site, oligonucleotide modifications that increase the melting temperature (T_m) of the oligonucleotide-RNA duplex, often increase antisense activity and/or potency (18–21). Mismatched oligonucleotides reduce the T_m and decrease the potency (22,23).

However, when comparing oligonucleotides targeted to different sites, T_m alone is not sufficient to ensure activity (3). It has long been believed that secondary structure in the mRNA target affects hybridization affinity differently at different sites and thus affects antisense efficacy (24–28). Therefore, methods for calculating RNA structure and calculating hybridization of the antisense oligonucleotide to the structured mRNA are useful for prediction of antisense activity.

Early attempts by Stull et al. (29) found moderate correlation (R = 0.66–0.99) between a predicted duplex score and antisense activity. Inclusion of an mRNA target secondary-structure score in the calculation actually worsened correlation between calculated hybridization affinity and antisense activity. Since Stull's publication, improvements have been made to the rules and parameters for prediction of RNA secondary structure. (30). Effective parameters for prediction of DNA:RNA duplex stability are available (31) and improved parameters for prediction of secondary structure in DNA oligonucleotides are also available (32–37). Mathews et al. (38) used these most up-to-date parameters to calculate equilibrium affinity of complementary DNA or RNA oligonucleotides to an RNA target taking into account the predicted stability of the oligonucleotide-target helix and the competition with predicted secondary structure of both the target and the oligonucleotide. When their predicted affinities were compared to antisense activity in one experiment (39), good correlation (R = 0.91) was found between duplex free energy and antisense activity. When oligonucleotide self-structure and/or target RNA structure were included in the calculation, antisense efficacy did not correlate with $\Delta G°_{overall}$.

The reported correlations between predicted duplex stability and antisense activity do not extend broadly to additional targets. When a data set of 349 antisense oligonucleotides targeting 12 genes (Giddings and Matveeva, *http://antisense.genetics.utah.edu*) was evaluated for correlation between duplex stability

and antisense activity, the linear correlation coefficient was 0.22, suggesting that the strong correlations reported in earlier work do not extend to larger data sets.

There are several possible explanations for the lack of a strong correlation between calculated hybridization of an oligonucleotide to its mRNA target and observed antisense activity. One possibility is that the calculated binding energies do not represent true equilibrium affinities. Although current algorithms are good enough to correctly predict 73% of base pairs in structures determined from comparative sequence analysis (30), this level of accuracy may not be enough to allow prediction of good antisense-binding sites. In addition, current algorithms (38) use thermodynamic parameters for unmodified DNA or RNA when calculating free energies of antisense: RNA duplex formation or antisense oligonucleotide self-structure. Parameters determined from experiments using modified oligonucleotides could improve the predictions (40). Furthermore, parameters for predictions were measured in 1 M Na^+, 0.1 mM EDTA, and may not represent conditions of antisense binding. The large numbers of proteins involved in RNA synthesis, processing, transport, translation, and degradation almost certainly affect binding of the antisense oligonucleotide to its target.

A second possibility is that the antisense target is pre-mRNA and secondary structures predicted for mRNAs are not representative of structures in pre-mRNAs. It is known that pre-mRNA is the molecular target for many antisense oligonucleotides (41,42). The secondary structure of a pre-mRNA undergoing synthesis, processing, and transport is likely not fully predictable from simple thermodynamic consideration.

The third, and most likely, possibility is that equilibrium affinity is not the sole factor impacting antisense activity (43). Oligonucleotide sequence and structure may affect properties of the antisense compound such as its affinity for proteins, ability to support RNAse H cleavage of the target, delivery to the cellular site of activity, and metabolic stability. These factors will, in turn, affect antisense activity. On the other hand, equilibrium affinity is not unimportant. When oligonucleotide sequence is kept constant, mRNA secondary structure affects antisense activity in a predictable way; activity is lower in structured targets than in unstructured ones (44).

Although factors other than target structure clearly play a role in antisense activity, predictions of local secondary structure have proven effective in identifying oligonucleotides with greater activity than those found by simple oligonucleotide "walks." The strategy employed by Sczakiel and colleagues (45,46) searches for favorable local target elements, loops or bulges of ~10 nt. joints and terminal sequences. Although successful application of this strategy to other targets has not yet been reported, it is tempting to speculate that the success is due to the fact these favorable local target elements represent kinetically preferred sites. "Kissing" hairpins are known to be important for initiation of hybridization

of long antisense RNAs (47,48); these "favorable structures" may play a similar role for oligonucleotide hybridization.

III. OLIGONUCLEOTIDE MOTIF PREFERENCES

It has been suggested that active oligonucleotides contain certain sequence motifs. Tu et al. (49) report that TCCC is associated with antisense activity but no mechanism for this phenomenon was proposed. Smetsers et al. (50) previously reported that CCC is overrepresented in the antisense oligonucleotides in their data set but that TCC is underrepresented. They suggest that overrepresented motifs may be associated with protein-binding and nonantisense effects. Lesnik and Freier (51) offered a plausible explanation for the predominance of pyrimidines and especially C's in active oligonucleotides. They suggests that antisense activity is associated with high stability of the oligo: target hybrid *relative to the alternative RNA:RNA duplex.* Thus antisense oligodeoxyribonucleotides with high (70–80%) pyrimidine content and moderate (40–50%) (A + T) content are more likely to be active than oligonucleotides with different composition.

IV. CELL-FREE SCREENING AND COMBINATORIAL APPROACHES

Several groups have described combinatorial approaches for identification of optimal antisense sites in target mRNA using a cell-free assay. Typically, a library of randomized oligonucleotides is incubated with the target mRNA and RNase H. Mapping of the most favored RNase H cleavage sites results in identification of the most favored binding sites. This approach has been used to find sites for both antisense oligonucleotides (39,52–54) and ribozymes (55). It can, however, be complicated by interactions of library oligonucleotides with each other and by binding of multiple oligonucleotides to the mRNA target (56). Concerns over library complexity have limited oligonucleotide lengths in these studies to 10 nt. Optimal binding sites for short oligonucleotides may not predict those for longer, antisense oligonucleotides.

Matveeva et al. (57) were able to use longer oligonucleotides and reduce library complexity by restricting the oligonucleotide pool to oligonucleotides complementary to the mRNA target sequence. A similar, but less thorough screen was performed by Jarvis et al. (58), who used a cell-free RNase H assay with individual oligonucleotides to identify optimal sites for synthetic ribozymes.

Optimal binding sites have also been identified without using RNase H cleavage assays. Ecker et al. (13) screened randomized combinatorial libraries of 2′-*O*-methyl- and phosphorothioate-modified compounds and identified com-

pounds that bind to H-*ras* mRNA. Using oligonucleotide arrays on glass slides, Southern and colleagues (59,60) were able to identify compounds that bound tightly to c-raf mRNA and were able to select the site for ISIS 5132, the most potent c-raf antisense compound reported at that time. Their synthetic approach uses an elegant strategy that results in synthesis of only oligonucleotides complementary to the mRNA of interest.

The effectiveness of these cell-free approaches requires that the most favored site(s) for oligonucleotide binding to the mRNA in the cell-free system will be the target site for the most active antisense oligonucleotide. To test whether this was the case, Matveeva et al. (61) evaluated the correlation between activity in an RNase H mapping assay or a gel shift binding assay with antisense activity in cells. Moderate correlation with cellular activity ($R = 0.6$) was found for both cell-free assays. Similar correlation analysis of the randomized library data of Ho (39,52) and the array data of Mir (62) gave coefficients of correlation between activity in the cell-free assay and antisense activity ranging from 0.2 to 0.7 (O. Matveeva, J. Wyatt, and S. Freier, unpublished). Thus the correlation between activity in the cell-free assay and antisense activity is relatively weak.

Despite the relatively weak correlation observed between oligonucleotide binding in the cell-free assay and antisense activity, ribozymes (55) or antisense oligonucleotides (39,52,54,57) designed to sites identified by combinatorial selection were more likely to be active than those selected without initial cell-free screening. Thus these methods can improve the "hit rate" for antisense discovery. However, these methods are cumbersome and, at best, result in several leads that still need to be screened in a cell-based assay. Therefore, the benefit of improved hit rate may not make up for the substantial cost disadvantage associated with these cell-free combinatorial assays.

V. SPECIFICITY CONSIDERATIONS AND NONANTISENSE EFFECTS

A. Target Specificity

One attraction of antisense technology is that high specificity can be achieved. For example, inhibition of one isoform of a protein can be obtained without affecting another (11,63,64). Such specificity is hard to achieve with small-molecule drugs. To obtain such specificity, one must be careful to design antisense oligonucleotides that will not hybridize to related mRNA sequences (65). Since oligonucleotides with as few as three mismatches are reported to be inactive (23), three mismatches to related targets should be sufficient but more would be desirable.

Unfortunately, the most commonly used tool for identification of sequence

homology, BLAST (66), is ineffective at finding mismatched sites for oligonucleotides. This is because the default window size is 11, meaning that there must be 11 matches in a row for BLAST to find the homology. The window size can be set as small as 7, but even then 20-mers with two mismatches (for example, in positions 7 and 14) are not found. A more effective technique for finding mismatched sites is to use BLAST to identify other mRNA sequences with homology to the target of interest and then use a substring search to find mismatched sites in these mRNAs. Sites with zero or a few mismatches should be avoided.

B. Motifs that Support Nonantisense Effects

Nonantisense effects of G-rich phosphorothioate oligonucleotides are well known (13,14) and have been attributed to the tendency of these oligonucleotides to form G-quartet structures that then interfere with biological processes (67). The simplest way to avoid these effects is to avoid G-rich oligonucleotides. Restricting oligonucleotides to less than 50% G with no G_4 strings and at most one G_3 string usually does not detrimentally limit the number of oligonucleotides that can be selected from a target message.

Homopolymers of other sequences also form unusual structures (68). Although nonantisense effects of these structures are not well characterized, this should be considered when designing oligonucleotides rich in any single nucleotide or containing strings of any single nucleotide.

Other motifs are also reported to produce nonantisense effects. Krieg et al. (15) reported that oligonucleotides containing CG, especially those with RRCGYY, can stimulate murine B cells in vitro and in vivo. The active motif in human cells is GTCGTT (69). To avoid designing any oligonucleotides containing the dinucleotide CG is, however, an overly stringent requirement. It eliminates nearly half the possible oligonucleotides that hybridize to a typical message from consideration, many of which show no immune stimulation at all. Therefore, it may be more prudent to avoid oligomers with the consensus hexamer motifs or to restrict the number of CGs in the sequence to less than two. In addition, the immunostimulatory effects of CG motifs are easily eliminated by chemical modification (e.g., 5-methyl C) (70).

VI. OTHER CONSIDERATIONS

A. Cross-Species and Cross-Isoform Oligonucleotides

One feature of antisense inhibitors is that usually an active inhibitor of the human target is not an inhibitor of the same gene in mouse or another species. This is because mRNA sequences differ between species. It is sometimes possible, how-

ever, to select sites with high identity between two species and design oligonucleotides to those sites. If a sufficient number of such sites are tested, it may be possible to identify an antisense oligonucleotide with activity in both species. Similarly, if sufficient sequence identity exists between two isoforms, it may be possible to identify an antisense oligonucleotide with activity against both targets. Using this strategy an oligonucleotide with good activity against both JNK-1 and JNK-2 was identified (71).

B. Target Site Function

The preceding discussion has considered RNA secondary structure at the target site and oligonucleotide sequence but has not seriously addressed position of the target site on the mRNA relative to functional sites such as the coding region. This is because antisense oligonucleotides that operate by an RNAse H mechanism seem to be affected little by target site function. Potent oligonucleotides have been reported for the coding regions, untranslated regions, and even introns. On the other hand, antisense oligonucleotides that use a non-RNase H mechanism are typically restricted to specific functional sites. Morpholino oligonucleotides, for example, inhibit via translation arrest and are often located near or upstream of the AUG initiation codon (72). They can also inhibit splicing if placed at splice junctions (21). Thus target site function becomes more important if a "steric blocking" mechanism of action is employed.

VII. SUMMARY

Design of antisense oligonucleotides is, in principle, very simple. In practice, on the other hand, only a fraction of antisense oligonucleotides complementary to an RNA target are active. Computational predictions of hybridization affinity that take into account RNA target structure, oligonucleotide self-structure, and oligonucleotide-RNA hybridization have had limited success at identifying potent antisense sites. Cell-based screening of a number of compounds is still required. Combinatorial approaches offer the potential of finding the best antisense oligonucleotide for any target. These approaches have not, in general, identified compounds with substantially greater activity than those designed by more conventional methods. In addition, significant effort is required for the cell-free screen and several compounds must still be screened in cell-based assays. Although no single approach has yet provided a method for identifying the single best target site for an antisense oligonucleotide, several guidelines are listed here that may improve "hit rates" and avoid screening of compounds likely to have nonantisense activities.

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Suborgan Pharmacokinetics

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I. INTRODUCTION

Antisense oligonucleotides are a promising new class of therapeutic agents designed to specifically and selectively alter the metabolism of RNA (1,2). The efficacy and safety of phosphorothioate oligodeoxynucleotides, the first generation of these compounds, in various animal models and in the clinical setting have been well documented in numerous publications (3–8). A great deal of information has also been gathered over the past 10 years concerning the basic pharmacokinetics of phosphorothioate oligodeoxynucleotides in vitro (9,10), in a variety of animal models and from human clinical trials (11–15).

As an earlier chapter in the book describes in vivo pharmacokinetics, we will only briefly summarize the pharmacokinetics of phosphorothioate oligodeoxynucleotides. After systemic administration, these drugs, which bind extensively to serum proteins, are rapidly cleared from blood, and distribute broadly to most peripheral tissues including liver, kidney, spleen, lung, lymph nodes, muscle, and bone. Liver and kidney represent the principal sites of accumulation (1,11–15). These compounds do not cross an intact blood-brain barrier. Although phosphorothioates are generally more stable in vitro and in vivo than their phosphodiester congeners, they are still labile and are cleared principally by metabolism through degradation by endo- and exonucleases present in serum and tissue (1,11,14,16). The aggregate data suggest that the absorption, tissue distribution, and metabolism of phosphorothioate oligodeoxynucleotides are similar across species, including mouse, rat, dog, and monkey, and independent of both route of systemic administration and oligonucleotide sequence (1,11,12,14).

nucleotides directed against a variety of disease and viral targets (6,8,12,52). Although data concerning absorption, plasma pharmacokinetics, and metabolism are available, it is obvious that studies examining suborgan distribution in humans are problematic. With these inherent limitations in mind, we compared the ex vivo uptake of 5′-fluorescein-coupled ISIS 1082 in primary human, monkey, and mouse hepatocytes following their attachment on collagen coated matrices (53–56). Over the 8-h experimental time period, cells consistently displayed greater than 90% viability, as assessed by trypan blue exclusion. Fluorescence microscopy (Fig. 8) revealed that primary hepatocytes of all three species internalized oligonucleotide in a similar fashion. In general, uniform cytoplasmic and nuclear staining was observed in all heptocytes. Interestingly, internalization of the conjugated oligonucleotide into these hepatocytes was accomplished without the need for cationic lipids, which are typically required to observe significant uptake of many oligonucleotides in some transformed tissue culture cell lines (10).

Experiments designed to quantitatively assess the proportion of oligonucleotide localized within subcellular compartments were also performed (Fig. 9). Following ex vivo treatment with 1 μM ISIS 1082, primary human hepatocyte nuclear, cytosolic, and membrane drug levels were measured over an 8-h period. Results from these experiments were compared against systemically administered drug from in vivo rat and mouse kinetic experiments. Oligonucleotide, once again, was extracted from subcellular isolates and subjected to CGE analysis (24). In general, human primary hepatocytes displayed a fourfold greater proportion of nuclear-associated oligonucleotide relative to mouse nuclei. At the 10 mg/kg dose level, no rat nuclear-associated drug was evident, as previously described. Another significant difference between human and rodent hepatocytes is the redistribution of oligonucleotide from the nuclear to cytosolic compartments in humans over 8 h.

Although no direct human-to-primate or rodent in vivo suborgan comparisons could be made, the indirect measures described above give some indication that human hepatocytes internalize phosphorothioate oligodeoxynucleotides and that a substantial portion of drug is distributed to the nuclear compartment. This could have a significant influence on the pharmacological effects of liver-specific antisense oligonucleotides if the target was localized within the nucleus.

IV. CONCLUSIONS

With the experimental caveats mentioned in Section II. D. and further complications in data analysis due to the different localization techniques, oligonucleotide backbones, lengths, sequences, doses, and rodent species and strains, a number of conclusions and generalizations are supported by the data. The most obvious conclusion derived from all of the studies is that phosphorothioate oligodeoxy-

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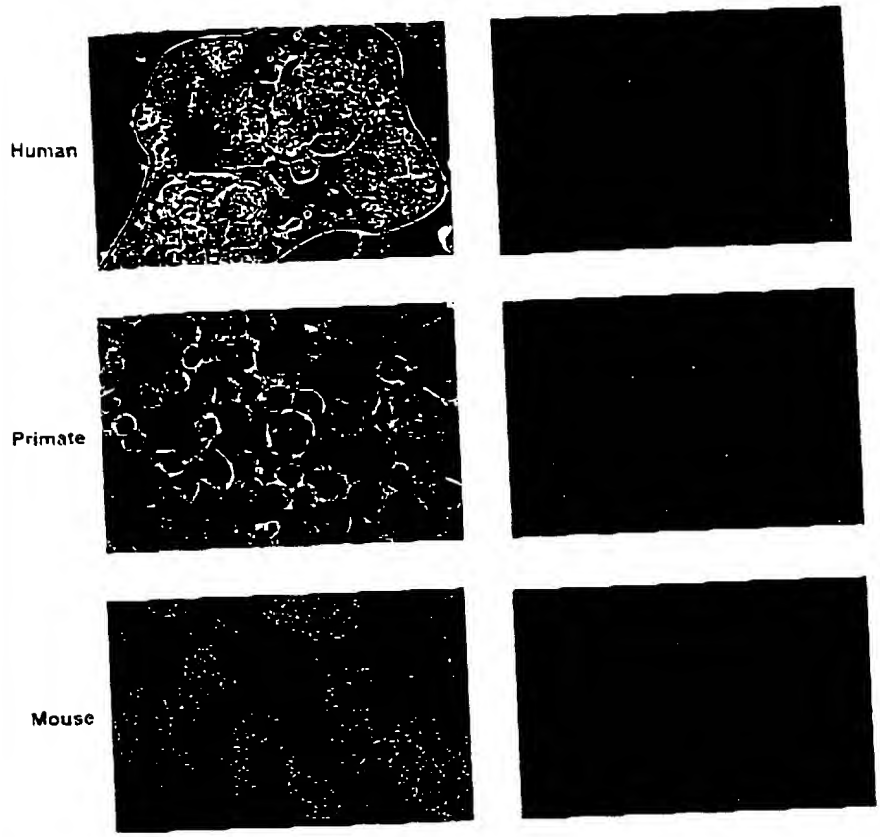

Figure 8 Fluorescent micrographs obtained following treatment with 5′-fluorescein-conjugated ISIS 1082. Primary human, monkey, mouse, and rat hepatocytes were isolated following collagenase perfusion and plated on collagen coated plates. Approximately 1 h after cell adherence, oligonucleotide was added at a 1 μM final concentration. This composite represents the fluorescence intensity observed after 8 h of treatment. (Left) Phase-contrast images of trypan-blue counterstained cells. (Right) Fluorescent micrographs obtained from the same field of cells.

nucleotides are unequivocally localized within the cells of organs, including liver, kidney, skin, spleen, and lymph nodes in a variety of animals after systemic administration of drug. Importantly, the broad distribution of these drugs in vivo has occurred without the need for special delivery systems involving complexation of oligonucleotides to substances like polycations or encapsulation within liposomes (57).

The second observation is the consistency of oligonucleotide localization

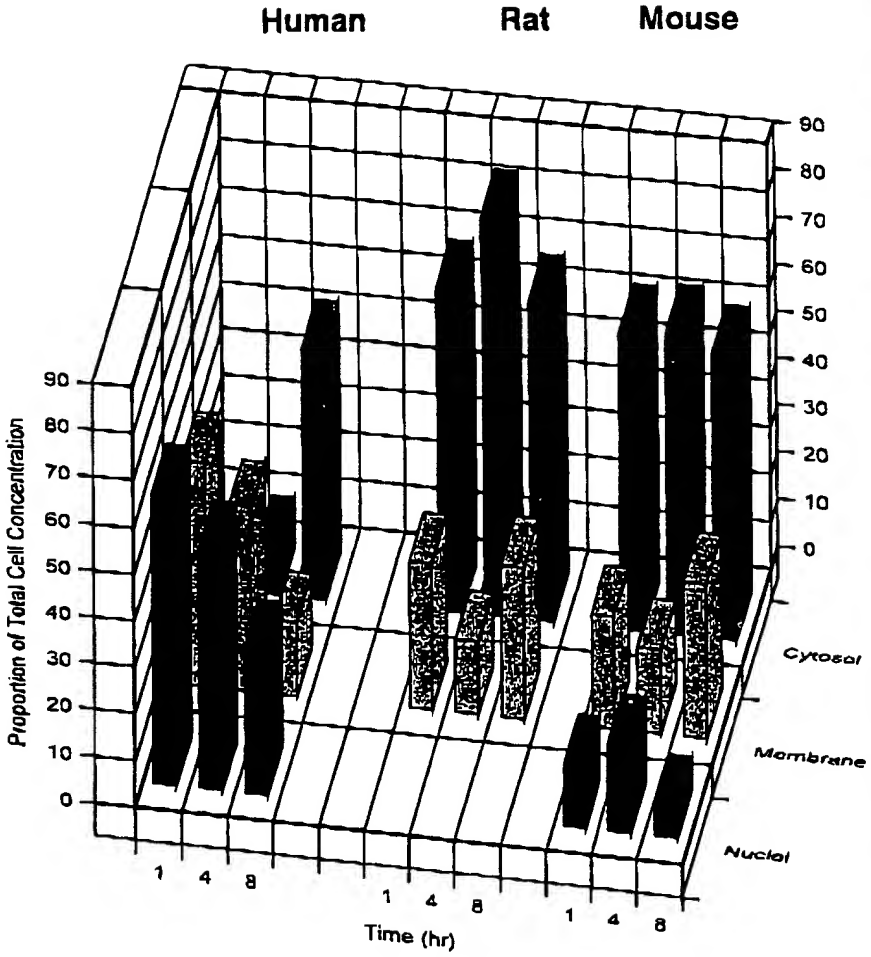

Figure 9 Effect of time on the proportion of ISIS 1082 found within nuclear, cytosolic, and membrane fractions isolated from human, rat, and mouse hepatocytes over an 8-h period after dosing. Results from human cells were obtained from ex vivo experiments whereas rodent results were produced following systemic delivery in vivo. Data are expressed as the proportion of the total cellular dose measured.

in those organs that accumulate the highest amounts of drug. For example Nicklin (11), Butler (17), Carome (18), Oberbauer (19), Plenat (20), Rappaport (21), and Rifai (22) all detected high concentrations of oligonucleotides in kidney in the renal proximal tubules, while some also localized drug to Bowman's capsule and the brush border (11,21,22). In liver, using a variety of indirect histochemical techniques, the highest concentrations of oligonucleotides were frequently detected within the cytoplasm of the nonparenchymal or Kupffer and endothelial cells (11,20,22). However, in some cases, drug was detected, albeit in lower concentrations, in hepatocytes (20,24).

In our laboratories, using a variety of detection methods, the suborgan and subcellular distribution and metabolism of phosphorothioate oligodeoxynucleotides has been carefully delineated and quantitated in mouse, rat, and monkey liver after systemic administration and primary human hepatocytes maintained in tissue culture. As described earlier, phosphorothioate oligodeoxynucleotides were localized within the nuclear, cytosolic and membrane compartments of all three liver cell types. Distribution varied as a function of time, dose, and species. HPLC/ES-MS analysis of liver samples also indicated the types of nucleolytic enzymes involved in the degradation of these compounds.

Based upon the aggregate data, it is also clear that localization data derived from a single technique, especially histological procedures, without validation with alternative methods, like CGE or HPLC/ES-MS for quantifying the amount and extent of metabolism, are incomplete and may provide misleading information. In our laboratories, we routinely perform both histological and subcellular fractionation and quantitative CGE to follow the organ and suborgan distribution of phosphorothioate oligodeoxynucleotides used in pharmacology and pharmacokinetic studies. We agree with Nicklin et al. (11) that multiple analytical techniques, including quantitative CGE, provide much more useful and complete pharmacokinetic information about the in vivo disposition of the phosphorothioates.

The in vivo suborgan localization experiments with phosphorothioate oligodeoxynucleotides, the first generation of antisense therapeutics in clinical trials, set the stage for answering a variety of questions concerning the in vivo pharmacokinetics of these exciting new drugs. Although we know these compounds enter cells and subcellular compartments of numerous organs, what is the mechanism of uptake? It was proposed that scavenger receptors were the primary mechanism of oligonucleotide uptake in liver and other tissues and cells like macrophages. As described earlier, Butler and colleagues (39) demonstrated, using Class A scavenger receptor knockout mice, that scavenger receptors alone cannot account for the bulk distribution of phosphorothioate oligodeoxynucleotides into tissues and cells and suggested multiple mechanisms of uptake probably exist. Current experiments in our laboratories are investigating the involvement of other molecules in in vivo uptake (M. Butler, personal communication; 58).

Other questions relating to the influence of oligonucleotide chemistry, sequence, species, and disease state on suborgan and subcellular distribution are currently being addressed in our laboratories. For example, at present, there are no explanations for the species-specific differences in subcellular distribution that we have observed in liver (45,51). Preliminary data derived from comparative pharmacological studies examining c-*raf* mRNA levels between mouse and rat suggest that the differences in subcellular distribution in hepatocytes may explain why antisense potency is greater in mouse than rat (48; J. Johnston, personal communication). Additionally, since only liver has been characterized in such detail, it is possible that differences in pharmacokinetics are present within other organs, such as kidney, spleen, and the gastrointestinal tract. We are currently developing analogous approaches for those organs as well. Given the complex interactions between cells and interstitium in vivo, the ability to examine and quantitate the distribution of antisense oligonucleotides using CGE after subcellular fractionation within a variety of organs represents a significant advance that has broad applicability for pharmaceutical research.

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Pharmacokinetic Properties in Humans

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I. INTRODUCTION

The use of antisense molecules to inhibit the expression of disease-causing proteins represents a new paradigm in disease treatment. The use of targeted inhibitors of protein translation is expected to become commonplace. Human diseases already being treated with antisense inhibitors of translation in clinical trials today include inflammatory bowel disease, hepatitis C, various cancers, and psoriasis. Important metabolic diseases such as diabetes are only minimally behind. Furthermore, the use of antisense inhibition of specific gene products is increasingly recognized as a valuable tool to help scientists understand the human genome and to elucidate new targets for therapeutic intervention.

The first antisense oligonucleotides to enter clinical trials have been phosphorothioate oligodeoxynucleotides, in which a single nonbridging oxygen of the internucleotide linkage was replaced by a sulfur to render nuclease resistance. The next generation of molecules will enter human trials by the year 2001 and will contain further modifications such as 2′-methoxyethyl modifications for improved potency and tissue elimination half-life, and methylated C residues for reduced immune stimulatory qualities. In fact, the tissue half-life of these newer agents is expected to be approximately 5× longer than that of the first-generation compounds (1).

Along with improved potency comes the potential for administration by nonparenteral routes. Indeed, work is already fairly advanced toward oral delivery of these later-generation oligonucleotides. A solid dosage form for oral delivery

of antisense oligonucleotides may already be available by the time of this publication. This was not thought possible just a short time ago.

Longer tissue half-lives mean the potential for less frequent dosing, which reduces patient cost and improves compliance by whatever route of administration available.

In this chapter we summarize currently available data on human pharmacokinetics of antisense oligonucleotides. As data in humans are not yet available for the newer modified oligonucleotides mentioned earlier, the focus of this chapter is on phosphorothioate oligodeoxynucleotides. The majority of our discussion will concern the compounds and references listed in Table 1.

II. PHARMACOKINETICS

A. General Pharmacokinetic Behavior in Humans

After intravenous administration of oligonucleotides, the plasma concentration-time profile is generally monophasic (<1 mg/kg). Use of radiolabeled material revealed a biphasic plasma profile with a much longer apparent elimination phase (2,3). Pharmacokinetics has been characterized for several oligonucleotides in a number of clinical studies (Table 1). The differences in the pharmacokinetics observed in these published clinical studies are mainly due to different assay methods used. The oligonucleotide assays used to date to describe the pharmacokinetic properties of various oligonucleotide preparations in humans and the associated shortcomings of each method are described below.

Capillary gel electrophoresis (CGE) provides excellent resolution for parent oligonucleotide and metabolites shortened by one or more nucleotides (N-1, N-2, etc.) with N-1 resolution, CGE is frequently used to quantitate parent oligonucleotide and metabolites (3–7,23). Another method, SAX-HPLC, measures primarily parent oligonucleotide; however, it does not provide adequate resolution to differentiate parent oligonucleotide and its N-1 metabolite. Similarly, measurement using radioactivity does not differentiate parent oligonucleotide from its metabolite, and very frequently, the radioactivity does not represent the intact oligonucleotide. High-performance electrophoretic chromatography (HPEC) was used for the analysis of a 20-mer phosphorothioate oligonucleotide complementary to p53 mRNA [OL(1)p53,a,b]. However, it is not clear from these reports whether the method is specific to parent oligonucleotide.

From the studies cited in Table 1, plasma concentrations of oligonucleotide increase during short infusion, and C_{max} is generally observed at or near the end of infusion. Parent oligonucleotide is cleared rapidly from the circulation and animal data demonstrate that it is distributed into tissues. Plasma distribution half-life is in the range of 30–90 min depending on dose. Thus the clearance from plasma measured in clinical trials is a distribution phase, not terminal elimination.

Table 1 Antisense Phosphorothioate Oligodeoxynucleotides Evaluated in Human Pharmacokinetic Studies

III. CONCLUSIONS AND SUMMARY

The pharmacokinetics of phosphorothioate oligonucleotides in humans are dose dependent, sequence independent, equivalent on a dose per body weight basis across nonhuman and human species, and characterized by high protein binding. Differing pharmacokinetic profiles observed in various clinical studies are due in large part to the analytical technique used, and dependent upon measuring parent versus parent plus chain-shortened metabolites.

C_{max} is generally observed at the end of infusion and clearance of oligonucleotide from plasma is predominantly by tissue distribution. After continuous infusion, steady-state concentration is generally achieved within 24 h. AUC is dose dependent and there appears to be a saturable component in the disposition of oligonucleotide. Plasma half-life is in the range of 30–90 min, depending upon dose. Phosphorothioate oligonucleotides in human plasma are rapidly metabolized to chain-shortened oligonucleotides by exonucleases in a processive fashion starting at the 3′ end (N-1, N-2, N-3, etc.). This metabolism is largely completed within 30 min and is independent of dose or sequence. Parent compound is the major component seen in plasma at all time points. Urinary excretion appears to be a very minor route of elimination (less than 1% of parent administered dose).

Approximately 91–99% of phosphorothioates are protein bound, with the major species being albumin and α_2-macroglobulin. Binding to thrombin, factor H, and components of the intrinsic tenase complex has also been shown to occur, and may account for some of the toxicities observed at high doses in nonclinical safety studies. Finally, plasma pharmacokinetics for humans can be accurately extrapolated from other species, and in nonhuman primates plasma concentration and AUC are nearly equivalent on a mg/kg basis.

Newer-generation oligonucleotides with additional modifications and enhancements are just now entering the clinic, and will likely exhibit different pharmacokinetic profiles. These modified oligonucleotides coupled with delivery systems and novel formulations will likely permit more convenient or flexible dosing, further enhancing the attractiveness of antisense as therapeutic agents for human disease.

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In Vivo Modulation of N-*myc* Expression by Continuous Perfusion with an Antisense Oligonucleotide

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ABSTRACT

In this study we investigated the *in vivo* efficacy of continuous subcutaneous perfusion of unmodified phosphodiester oligodeoxynucleotides. The *in vitro* sequelae of antisense inhibition of the target gene, N-*myc*, have been documented and include moderate growth inhibition without effects on *myc* expression, loss of secretogranin I expression, and morphologic alterations. We chose to use N-*myc* as a model target to determine if antisense effects observed *in vitro* can be reproduced *in vivo*. N-*myc*-expressing human neuroectodermal tumors were grown as subcutaneous xenografts in athymic mice. Antisense and sense oligodeoxynucleotides directed against N-*myc* were delivered to the vicinity of the tumor by a subcutaneously implanted microosmotic pump. Antisense treatment led to loss of N-*myc* protein from the tumor, as well as to the loss of the neuroendocrine differentiation marker protein secretogranin I. *Myc* protein expression remained unaffected. Mean tumor mass was reduced by 50% in antisense-treated animals, and antisense-treated tumors morphologically resembled antisense-transfected *in vitro* cell cultures. These results demonstrate that regional, *in vivo* perfusion of an unmodified oligonucleotide specifically downregulates gene expression in human tumor xenografts with concomitant effects on tumor phenotype and growth rate that correlate well with *in vitro* observations.

INTRODUCTION

TRULY TUMOR-SPECIFIC CHEMOTHERAPEUTIC AGENTS that can selectively kill or inhibit the growth of cancer cells without damaging normal cells have yet to be found. Because it is becoming increasingly apparent that human cancers result from the stepwise accumulation of genetic abnormalities within a target tissue, inhibition of expression of a particular cancer-associated gene appears to be a rational approach to the development of tumor-specific therapy (McManaway *et al.*, 1990). Oligodeoxynucleotide-based antisense technology relies on the ability of short, single-stranded DNA molecules to inhibit, in a sequence-dependent manner, translation of specific messenger RNA molecules into protein. In the laboratory, antisense oligodeoxynucleotides (oligos) have repeatedly demonstrated efficacy in modulating the expression of numerous oncogenes, thus providing important insights into their unique roles in tumorigenesis (Cohen,

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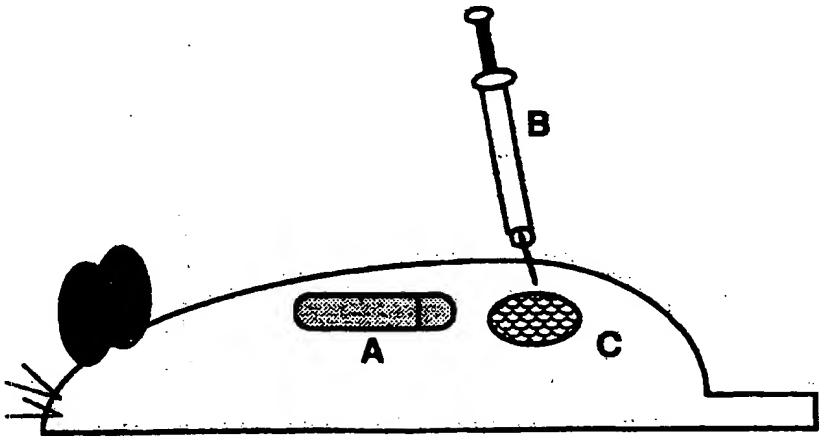

FIG. 1. Subcutaneous pump-tumor xenograft model for continuous *in vivo* infusion of oligodeoxynucleotides.

1989; Helene and Thuong, 1989; Uhlmann and Peyman, 1990; Neckers, *et al.*, 1992). Despite intensive study *in vitro*, however, the application of oligo antisense strategies to regulation of gene expression *in vivo* has yet to be demonstrated.

We previously demonstrated that administration to cells of either an unmodified N-*myc* antisense pentadecadeoxynucleotide or an antisense RNA-expressing episomal replicon specifically inhibits N-*myc* gene expression and decreases cell growth rate *in vitro* in the human neuroepithelioma cell line CHP-100 (Rosolen *et al.*, 1990; Whitesell *et al.*, 1991). In an effort to extend these observations to the modulation of protooncogene expression *in vivo* and to begin to explore the utility of antisense oligos as gene-targeted pharmacologic agents, we developed a human tumor xenograft model in which oligo solutions are continuously perfused via subcutaneously implanted microosmotic pumps. Such administration prevents degradation of bulk oligo while allowing the controlled, continuous release of relatively small quantities of drug into surrounding tissue. Using this model, we now describe the successful *in vivo* application of antisense oligonucleotides as gene-targeted pharmacologic agents.

MATERIALS AND METHODS

The human neuroepithelioma cell line CHP-100 was obtained from Children's Hospital of Philadelphia and has been well described (Schlesinger *et al.*, 1976). Cells were maintained in culture in RPMI 1640 supplemented with 10% fetal bovine serum. Male, athymic nude mice aged 4–6 weeks were obtained from the Frederick Cancer Research Facility and maintained under standard aseptic conditions.

Inoculation and oligo treatment of cells is illustrated in Fig. 1. Microosmotic pumps (Model 1007D; Alza Corporation, Palo Alto, CA) were filled with a 5 mM solution in water of either N-*myc* sense oligonucleotide (5'dATG CCG GGC ATG ATC-3') or antisense oligonucleotide (5'-dGAT CAT GCC CGG CAT-3') as specified by the manufacturer. These pumps continuously deliver 0.5 μl (2.5 nmol oligo) of their contents per hour. Standard phosphodiester oligonucleotides, synthesized using cyanoethylphosphoramidite chemistry, were obtained from Gilead Sciences (Foster City, CA) and Synthecell Corporation (Gaithersburg, MD) and were chromatographically purified. Pumps were implanted in pentobarbital-anesthetized athymic mice in a subcutaneous pocket formed by blunt dissection through a 0.5 cm transverse, paraspinal skin incision just caudal to the right forequarter (Fig. 1,A). CHP-100 cells were harvested from confluent monolayers 1 day later, and suspended in phosphate-buffered saline (PBS) at 1×10^8 cells per ml. The suspension was divided

into equal aliquots and made 100 μM in either N-*myc* sense or antisense oligonucleotide. The appropriate suspension (100 μl) was injected subcutaneously, caudal to the previously implanted pump (Fig. 1B). Pumps were replaced 7 days following implantation with fresh oligonucleotide-filled pumps. Animals were sacrificed 14 days after tumor cell injection, and the well-encapsulated tumors were easily resected for weighing, homogenization in protein lysis buffer, and/or snap freezing for frozen sectioning (Fig. 1C).

Immunoblot analysis of protein expression in oligo-perfused tumors was performed as previously described (Whitesell *et al.*, 1991), except that detection of primary antibody was achieved using the chemiluminescent alkaline phosphatase substrate AMPPD and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Western Light Kit; Tropix, Inc., Bedford, MA). Tumors were resected from pump-bearing animals, minced, and homogenized in lysis buffer at 100 μg wet tissue per ml. Tissue culture cells were harvested from monolayers and lysed in the same buffer at 2.5×10^7 cells per ml. N-*myc* expression was assessed using the mouse monoclonal antibody NMC II-100 (Ikegaki *et al.*, 1986). *Myc* was assessed with the human-specific monoclonal antibody 9E10 (Evan *et al.*, 1985), and murine monoclonal anti-secretogranin I was obtained from Boehringer Mannheim (Indianapolis, IN).

Immunocytochemistry was performed on vector-transfected cells as previously described (Whitesell *et al.*, 1991). Frozen sections of tumors from pump-bearing mice were fixed in methanol and stained with the Diff-Quik differential staining kit (Baxter Diagnostics, Scientific Products Division, McGaw Park, IL) for simple histologic evaluation.

RESULTS

A 2 week constant subcutaneous perfusion of unmodified phosphodiester oligos at the rate of 2.5 nmol/h resulted in no overt whole-animal toxicity (i.e., altered water or food intake, weight loss, or lethargy). N-*myc* gene expression in tumor xenografts from these oligo-perfused animals was assessed by immunoblotting. A marked reduction in N-*myc*-specific signal was observed in lysates of antisense-perfused tumors (Fig. 2A, lane 3) compared to lysates of sense-perfused tumors (Fig. 2A, lane 2). The lower migrating band seen in tumor lysates but not in tissue culture cell lysates presumably represents a murine protein(s) cross-reacting with the polyclonal secondary antibody. The decrease in N-*myc* expression following antisense perfusion is specific for the targeted gene, since as seen previously *in vitro* (Rosolen *et al.*, 1990; Whitesell *et al.*, 1991), expression of the closely related *myc* gene remains unchanged in samples of both sense- and antisense-perfused tumors (Fig. 2B, lanes 2 and 3). Because the *myc* antibody used for this blot does not cross-react with murine *myc* (Evan *et al.*, 1985), equivalent amounts of human tissue must have been lysed in sense- and antisense-perfused tumors. Thus, the decrease in N-*myc* signal signifies decreased gene expression, not simply cell loss or dilution by murine stromal components.

Many established neuroblastoma cell lines have been reported to be composed of at least two distinct morphologic types that display variant phenotypic characteristics and spontaneously interconvert or transdifferentiate (Ciccarone *et al.*, 1989; Rettig *et al.*, 1987; Ross *et al.*, 1983). We previously showed that the neuroepithelioma cell line used here, CHP-100, is also composed of distinct morphologic and phenotypic cell types (Whitesell *et al.*, 1991). Using vector-generated RNA antisense, we demonstrated that inhibition of N-*myc* expression blocks transdifferentiation in this cell line and results in accumulation of cells with an intermediate morphology and phenotype. Thus, expression of markers of neuronal differentiation, such as secretogranin I, are markedly diminished *in vitro* in N-*myc*-suppressed CHP-100 clones (Whitesell *et al.*, 1991). As Fig. 2C demonstrates, *in vivo* antisense inhibition of N-*myc* expression in tumor xenografts also results in loss of expression of secretogranin I.

N-*myc* suppression *in vivo* is also accompanied by striking changes in the gross histologic appearance of the tumor. Immunocytochemical analysis demonstrates the morphologic sequelae of N-*myc* inhibition *in vitro* by vector-generated RNA antisense (Fig. 3A and B) (Whitesell *et al.*, 1991). Note the loss of dark

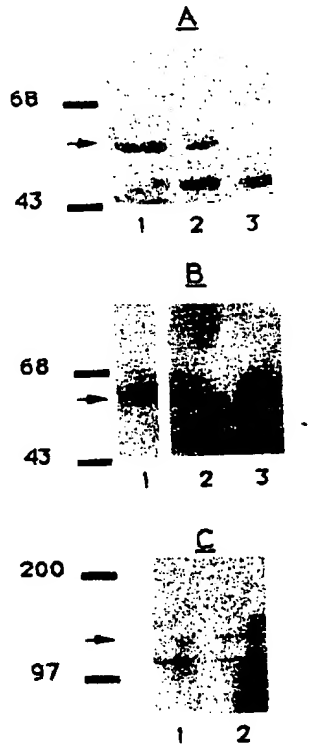

FIG. 2. Immunoblot analysis of protein expression by oligonucleotide-perfused tumors. (A) Blot was incubated with the N-*myc*-specific monoclonal antibody NMC II-100: lane 1: tissue culture cell lysate: lane 2. sense-perfused tumor lysate: and lane 3. antisense-perfused tumor lysate. (B) Blots were incubated with human *myc*-specific monoclonal antibody 9E10: lane 1. tissue culture cell lysate: lane 2. sense-perfused tumor lysate: and lane 3. antisense-perfused tumor lysate. (C) Blot was incubated with murine monoclonal antisecretogranin I: lane 1. antisense-perfused tumor lysate: and lane 2. sense-perfused tumor lysate. Panels A–C were obtained with the same lysates. Tissue culture lysates are included for qualitative. not quantitative comparison with the tumor lysates. Molecular weight markers (kilodaltons) are indicated for each panel. The protein of interest in each panel is indicated by an arrow.

N-*myc*-specific nuclear reactivity and the absence of small, condensed cells in antisense vector-transfected cells (Fig. 3B) compared to sense vector-transfected cells (Fig. 3A). Tumors from mice treated *in vivo* with antisense oligos show similar morphologic changes compared to tumors from sense-treated mice. Diff-Quik–stained frozen sections of a tumor harvested from an antisense-perfused animal show loss of small condensed cells and cells with processes (compare C and D in Fig. 3). This decrease in the proportion of small, densely stained cells is most readily appreciated as an apparent decrease in cellularity (all fields were photographed at the same magnification).

We showed previously that *in vitro* antisense inhibition of CHP-100 N-*myc* expression results in a decreased growth rate without a reduction in *myc* expression (Rosolen *et al.*, 1990). The decrease in growth rate is due to inhibition of a transdifferentiation process rather than to a direct tumoricidal or antiproliferative effect (Whitesell *et al.*, 1991). *In vitro*, cells in which vector-generated antisense completely suppressed N-*myc* expression (undetectable by western blot analysis) grew at 50% of the rate of sense- or sham-transfected cells. N-*myc*-suppressed cells also continued to form tumors in nude mice with good efficiency, but these tumors grew more slowly than those formed by nonsuppressed cells. Figure 4 demonstrates that

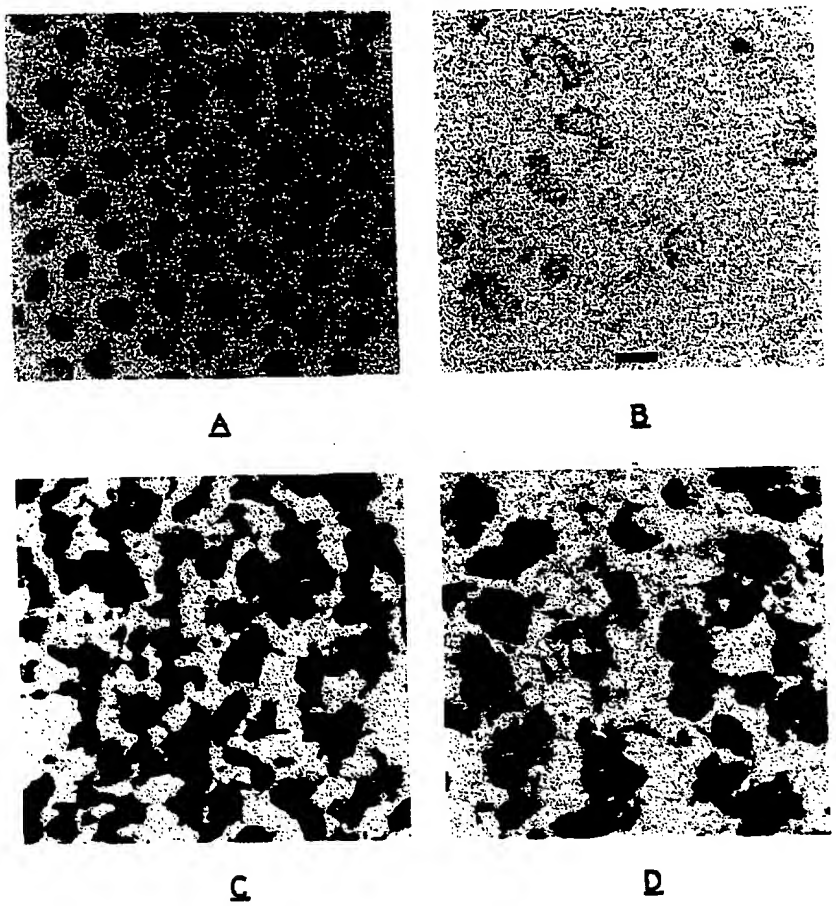

FIG. 3. Morphologic effects of N-*myc* suppression *in vitro* and *in vivo*. N-*myc*-specific immunocytochemistry was performed on vector-transfected CHP-100 clones (A and B). Frozen sections of tumors from pump-bearing mice were fixed in methanol and stained for simple histologic evaluation (C and D). (A) N-*myc* sense vector-transfected CHP-100; (B) N-*myc* antisense vector-transfected CHP-100; (C) sense oligonucleotide-perfused xenograft; (D) antisense-perfused xenograft. The bar in B (pertaining to all panels) represents a distance of 40 μm.

continuous perfusion of CHP-100 cells *in vivo* with antisense oligo also does not abrogate tumor formation, but instead leads to significantly smaller tumors. In three separate experiments, groups of four to five animals were implanted with tumor cells and microosmotic pumps loaded with either sense or antisense oligonucleotide. Animals were sacrificed on day 14 after cell inoculation, tumors were resected, and their wet weight was determined. In combined analysis of three separate experiments, the average weight of sense-treated tumors was 306 mg; the average weight of antisense-treated tumors was 150 mg. Remarkably, the 50% decrement in mean tumor mass observed in antisense-treated animals is nearly identical to the maximal growth inhibition observed *in vitro* following either antisense oligonucleotide administration or antisense vector transfection (Rosolen *et al.*, 1990; Whitesell *et al.*, 1991). A two-factor analysis of variance revealed that the groups were significantly different by treatment ($p = 0.03$); (i.e., when all three experimental groups were combined) but not by experiment ($p = 0.7$). In many control experiments in which animals received no treatment, tumor mass at 14 days postinoculation ranged from 300 to 350 mg, not significantly different from tumors excised from sense pump-treated animals. In several experiments in which tumors were not excised for

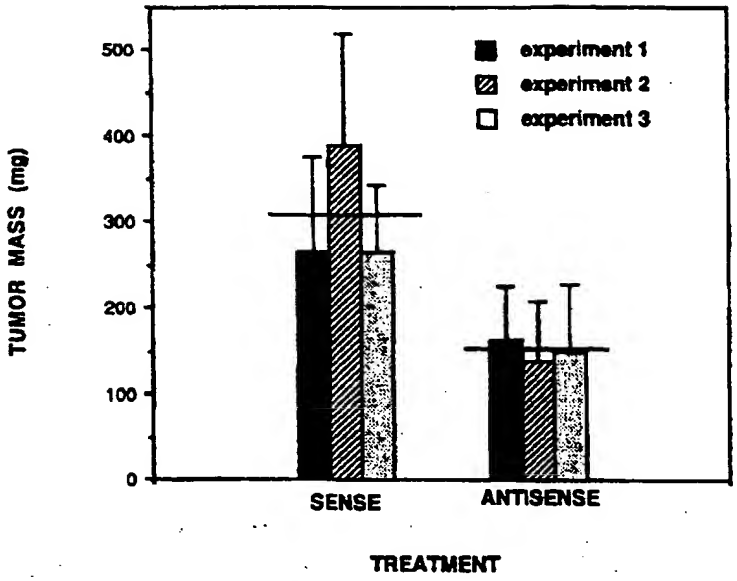

FIG. 4. Continuous *in vivo* perfusion of N-*myc* antisense oligonucleotide inhibits the growth of CHP-100 xenografts. Data are depicted as mean ± standard deviation. Each bar represents a separate experiment comprising four to five animals. In an analysis of variance the groups are significantly different by treatment ($p = 0.03$).

1 week following pump expiration, no significant difference was observed between tumor mass in sense- and antisense-treated animals, demonstrating that the effect on tumor growth was reversible (data not shown). At the same time, reappearance of N-*myc* protein was observed in these antisense-treated animals (data not shown).

DISCUSSION

The results presented here demonstrate that regional perfusion of an unmodified oligo in an animal can specifically modulate protooncogene expression with the same sequelae *in vivo* that are seen *in vitro*. Thus, not only does expression of the target gene decline (without decline in expression of a closely related gene), but the phenotype and biologic behavior of the perfused tumors are altered as well. Importantly, these alterations are achieved without overt local or systemic toxicity.

N-*myc* alone may not be an appropriate focus for further development of gene-targeted antitumor therapy, since its specific inhibition *in vivo* does not lead to loss of tumorigenicity or cell death. In concordance with a multihit hypothesis of oncogenesis, it is likely that the interplay of several positive and negative oncogenes is required to maintain the tumorigenicity of CHP-100. Simultaneous, or even sequential inhibition of such multiple genes could lead to loss of the tumorigenic phenotype. As our understanding develops of the precise role played by various growth-associated genes in the malignant phenotype, more of these targets are likely to become apparent.

Successful systemic administration of oligos has been hindered by at least three prominent technical difficulties: (1) rapid degradation of unmodified phosphodiester oligos within biologic fluids (Goodchild *et al.*, 1991); (2) rapid clearance from the circulation (Goodchild *et al.*, 1991; Chen *et al.*, 1990; Agrawal *et al.*, 1991); (3) overwhelming cost associated with synthesis of the pharmacologic quantities required for systemic treatment of animals (Zon, 1989). Add to these considerations the observation that oligo uptake is a

property of most if not all cells (Loke *et al.*, 1989; Yakubov *et al.*, 1989; Neckers, 1989), and it is apparent that systemic delivery of a nontargeted antisense reagent in sufficient concentration to its intended site is currently a very difficult task. Given the relatively low potency of unmodified antisense oligos, certain antisense-based therapeutic strategies requiring systemic administration may not prove effective, even if stability and bulk production problems can be overcome. Furthermore, systemic administration of DNA, modified to enhance stability or cellular uptake, must be viewed with particular caution, since eventual degradation of these oligos could lead to incorporation of modified nucleotides into cellular DNA, with a significant potential for mutagenicity. This led us to test the *in vivo* efficacy of unmodified phosphodiester oligos in the model presented here.

We sought to overcome some of the obstacles associated with *in vivo* admininstration of oligos by avoiding the need for their systemic application. Our data suggest that the current generation of oligos can be developed as clinically efficacious therapies in well-defined clinical settings in which systemic administration is not required. Localized continuous perfusion of antisense oligos effectively modulates tumor cell gene expression in an animal to a similar degree to that observed *in vitro*. In addition, the sequelae of N-*myc* inhibition observed *in vitro* have been reproduced in these experiments, supporting a role for N-*myc* in the differentiation and growth of neuroectodermal tumors *in vivo*.

The model we describe can be useful for *in vivo* efficacy screening of both new target sequences and novel oligo modifications, as well as for determining *in vivo* toxicities of antisense reagents. In addition, this model can be used to test the role of target genes in tumor growth *in vivo*. For example, our current data clearly demonstrate that sole inhibition of tumor cell N-*myc in vivo* is not sufficient to abrogate tumorigenesis. At the same time, other clinically relevant models suggest themselves. Studies are underway exploring the utility of intrathecal perfusion of oligos for the treatment of leptomeningeal tumors in the athymic rat and topical administration to hyperproliferative epidermis in a murine model of psoriasis.

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In vitro and ***in vivo*** pharmacologic activities of antisense oligonucleotides

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Summary: The use of antisense oligonucleotide as pharmacologic agents is a derivative of the central dogma of molecular biology and knowledge of the physical and chemical properties that govern the structure of nucleic acids. Oligonucleotides have been reported to inhibit the growth of a large number of viruses in cell culture, as well as the expression of numerous ocogenes, a variety of normal genes and transfected reporter genes controlled by several regulatory elements. The therapeutic activity of antisense compounds in animal disease models have also been reported.

This review provides some general conclusions and trends regarding the pharmacologic action of antisense oligonucleotides, that can be formulated from studies previously reported in the literature. In addition, data is highlighted for two specific examples in which antisense oligonucleotides have demonstrated activity against herpes viruses and intracellular adhesion molecule RNA targets.

Introduction

In the past few years, many papers have been published demonstrating the activity of numerous antisense oligonucleotides, of different sequences and chemical type, in a variety of cell-based systems. Recently there have been a number of excellent reviews that have summarized the activities of these compounds in detail (Cohn, 1989; Uhlman & Peyman, 1990; Cazenave & Helene, 1991). As such this review will not attempt to duplicate those comprehensive efforts; instead it will provide a brief summary of the activities of oligonucleotides in cell-based assays and attempt to provide some general conclusions and trends that can be formulated from these previously published data. In addition, this paper will provide examples of data compiled in our laboratories that relate to the pharmacological activities of phosphorothioate oligonucleotides directed against cellular and infectious disease targets.

Pharmacological activities in cell-based models

Oligonucleotides have been reported to inhibit the growth of a large number of viruses in cell culture, as well as the expression of numerous oncogenes, a variety of normal genes and transfected reporter genes controlled by several regulatory elements. These studies varied in the types of oligonucleotides used, the cells used, the RNAs and specific receptor sequences targeted and the conditions employed. Although a wide range of oligonucleotide concentrations have been used to treat cells, only a few studies have reported detailed dose-response curves and clearly documented the purity of the oligonucleotides used. Table I summarizes the information from more than 40 papers in which oligonucleotides were tested for pharmacologic

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Table I Summary of cell-based *in vitro* activities of antisense oligonucleotides

Target viruses	*Cell type*	*Serum*	*Oligo types*	*Oligo length*	*Effective oligo concentration*	*References*
HTLV-III	H9 cells	–	P	12–26	5–50 mg/ml	Zamecnik *et al.* [1986]
HIV	H-T cells	+	P-S	14–28	0.5 μM	Matsukura *et al.* [1987]
HIV (gag/pol)	H-T cells	+	P-S	18–24	1–10 μM	Kinchington & Galpin [1989]
HIV	H9 cells	+	PS, others	20	4–20 μg/ml	Agrawal *et al.* [1988]
HIV	CZM cells	+	PS	18–28	10 μM	Vickers *et al.* [1991]
Herpes simplex	Vero cells	+	CH3-P	7	50–100 μM	Smith *et al.* [1989]
Herpes simplex	HeLa cells	+	PS	28	1–10 μM (non-antisense)	Gao *et al.* [1989]
Herpes simplex	Vero cells	+	CH3-P	12	20–50 μM	Kulka *et al.* [1989]
Herpes simplex	Vero cells	+	CH3-P-psoralen	12	5 μM	Kulka *et al.* [1989]
Herpes simplex	HeLa cells	+	PS	21	0.2–4 μM	Draper & Brown-Driver [1991]
Vesicular stomatitis	L929 cells	+	CH3-P	9	25–50 μM	Agris *et al.* [1986]
Vesicular stomatitis	L929 cells	+	P-lipid	11	50–150 μM	Shea *et al.* [1991]
Vesicular stomatitis	L929 cells	+	p-poly l-lysine	10–15	0.1 μM	LeMaite *et al.* [1987]
Influenza	MDCK cells	+	P-acridine	11	50 μM	Zerial *et al.* [1987]
Tick borne encephalitis	—	+	Various	Various	0.1–1 μM	Vlassov [1989]
SV40	MDCK cells	+	CH3-P	6–9	25 μM	Miller *et al.* [1985]
Rous	Chicken fibroblasts	+	Various	Various	10 μM	Zamecnik & Stephenson [1978]
Hepatitis B	Alexander	+	P	15	8.5 μM	Goodarzi *et al.* [1990]
Bovine papilloma virus	C-127 cells	+	PS	4–30	0.01–1 μM	Cowsert & Fox [1991]

Oncogenes						
c-myc	T-lymphocytes	+	P	15	30 μM	Heikkila *et al.* [1987]
c-myc	HL-60 cells	+	P, PS	15	10 μM	Wickstrom *et al.* [1989]
c-myc	Burkitt cells	–	P	21	100 μM	McMananway *et al.* [1990]
c-myb	PMBC	+	P	18	40 mg/ml	Gewirtz *et al.* [1989]
BCL-2	L697 cells	–	P ± PS	20	25–150 μM	Reed *et al.* [1990]
N-ras	T15 cells	+	CH3-P	9	Inactive	Tidd *et al.* [1988]
Host genes						
Multiple drug resistance	MCF-1 cells	+	PS	15	—	Jaroszewski *et al.* [1990]
PCNA (cyclin)	3T3	+	P	18	30 μM	Jaskulski *et al.* [1989]
Prothymosin	Human myeloma cells	–	P	16–21	40 μM	Sburlati *et al.* [1991]
T cell receptor	T cells	+	P	22	—	Zheng *et al.* [1989]
Gm CSF	Endothelial cells	–	P	15, 18	10^{-5} M	Zheng *et al.* [1989]
CSF-1	FL-ras/myc cells	+	P	—	—	Birchenall *et al.* [1989]
EGF receptor		+	P	13	30 μM	Yeoman *et al.* [1989]
β Gl bin	Rabbit reticulocytes	+	CH3P	9	100 μM	Blake *et al.* [1985]
cAMP-protein kinase II_β	HL-60	+	P	21	15 μM	Tortora *et al.* [1990]
Phospholipase A2 Activating protein	BC3H1	+	P	25	25 μM	Clark *et al.* [1991]
bFGF	Human astrocytes	–	P	15	10–75 μM	Morrison [1991]
TAU	Neurons	–	P	20–25	3–50 μM	Caceres & Kosik [1990]
Myeloblastin	HL-60 cells	+	P	18	—	Bories *et al.* [1989]
ICAM-1	A549, HUVEC	–	PS	18–20	0.01–1 μM	Chiang *et al.* [1991]
IL-2	T lymphocytes	–	P	15	5 μM	Harel-Bellan *et al.* [1988]
IL-1α	HUVEC	+	P	18	10 μM	Maier *et al.* [1990]
IL-1β	Monocytes	+	PS	15	0.1–2.5 μM	Manson *et al.* [1990]
IGF-1	Myoblasts	–	P	15	10 μM	Florini & Ewton [1990]
Perforin	T lymphocytes	–	P	18	5–35 μM	Acha-Orbea *et al.* [1990]

Table I (*contd.*)

Target	*Cell type*	*Serum*	*Oligo types*	*Oligo length*	*Effective oligo concentration*	*References*
Other						
Chloramphenicol acetyl transferase	CV-1 cells	+	P, PS, CH3P	21	5–30 μM	Marcus-Sekura *et al.* [1987]
Placental alkaline Phosphatase driven by HIV TAR	SK-mel-2 cells	+	PS	18–28	0.25–5 μM	Vickers *et al.* [1991]
Chloramphenicol acetyl transferase driven by human papilloma virus E2 responsive element	C-127 and CV-1 cells	+	PS	14–20	1–10 μM	Cowsert & Fox [1991]

cAMP = cyclic AMP; EGF = epidermal growth factor; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte macrophage colony-stimulating factor; HB = hepatitis B; HIV = human immunodeficiency virus; HSV = herpes simplex virus; HTLV = human T cell lymphotrophic virus; IV = influenza virus; PCNA = proliferating cell nuclear antigen; RSV = Rous sarcoma virus; TAR = TAT response element; TBE = tick-borne encephalitis; VSV = vesicular stomatitis

activities against a variety of viruses, oncogenes, host genes and transfected reporter genes.

The data presented in Table I support only a few generalizations. First, while phosphodiesters are rapidly degraded in biological systems, a number of investigators have reported activities for unmodified phosphodiester oligonucleotides in cells incubated in the absence of serum or in medium supplemented with heat-inactivated serum. When phosphodiester oligonucleotides have displayed activity, concentrations of more than 10 μM were required. The explanation for these activities is unclear. Considering the presence of endo- and exonucleases that are found within cells it is reasonable to think that these oligonucleotides would be degraded in the cell very rapidly. Evidence from our laboratory demonstrates that in a number of routinely used cell lines phosphodiester oligonucleotides are degraded within minutes by nucleases found in the plasma membrane, cytoplasm and in nuclei (Hoke et *al.*, in press).

Second, a variety of chemically modified oligonucleotides have been reported to be active in cell culture. Although considerable variation has been reported, phosphorothioate oligonucleotides appear to be more potent than methylphosphonate oligonucleotides. Conjugation of alkylators and interchelators to phosphodiesters and methylphosphonates has been reported to increase potency. Many of these modifications have been positioned at either the 3' or 5' end of the oligonucleotides; 3' positioning is an attempt to increase stability to 3'-exonuclease, the predominant serum nuclease. Lipophilic and poly(L-lysine) conjugates have also displayed enhanced potencies presumably via some modulation of cellular pharmacokinetic characteristics.

Third, oligonucleotides have demonstrated activities against a broad array of viral targets, oncogenes, normal cellular gene products and various transfected genes. This array of pharmacological effects clearly demonstrates the broad potential therapeutic applicability of these drugs.

Fourth, although the data from studies included in Table I are limited, when it is combined with in vitro toxicologic data (Crooke, 1991), the therapeutic indexes of phosphorothioate oligonucleotides appear to be quite high. Initial data regarding certain phosphorothioates of 20 and 21 nucleosides in length, targeted to human papilloma virus and herpes simplex virus, respectively, also demonstrate that these compounds are extremely well tolerated in animals (Mirabelli et *al.*, in preparation). The effects of specific base composition within an oligonucleotide, oligonucleotide length, specific chemical modifications in oligonucleotide and cellular parameters (i.e. cell type, cell cycle phase and stages of differentiation) on the potential toxicology and non-antisense activities of these compounds are not yet clearly defined (Crooke, 1991).

Fifth, very little data that support putative mechanisms of action have been reported and generalizations concerning precise mechanisms of action are not possible. A variety of mechanisms have been proposed to explain the ultimate pharmacologic action of antisense oligonucleotides, all resulting from the hybridization of the drug with the complementary sequence within a target RNA. These mechanisms include the disruption of ribosomal assembly and function, formation of an RNase H substrate and subsequent cleavage of the target RNA, and disruption of RNA splicing processes or other RNA metabolic processes. It is very likely that many 'terminating' mechanisms can be exploited for the cellular action of antisense oligonucleotides and that the mechanisms of a particular oligonucleotide are the result of the particular RNA and sequence target, the cell in which the drug is. acting and the chemical structure of the oligonucleotides.

Examples of antisense pharmacologic activities

Our laboratory has demonstrated activities of oligonucleotide drugs against a number of molecular disease targets. Below is a brief summary of work on two targets: herpes simplex virus and a human cell adhesion molecule, ICAM-1. These data are reviewed in an attempt to provide examples of the antisense drug discovery process and the activities of antisense compounds directed against viral gene targets and host gene targets.

Antisense oligonucleotides directed to herpes simplex virus RNA targets

In vitro activities. Smith & Smith (1986) first reported antisense inhibition of HSV replication using oligonucleotides targeted to the splice junction sequences of the HSV-1 1E4 and 1E5 pre-RNAs. It was later reported that increasing the length of the oligonucleotide increased the antiviral activity against HSV-1 (Kulka *et al., 1989).* The most active oligonucleotide, a 12-nucleotide long oligomethylphosphonate, was directed against a splice junction covering six nucleotides in both exon and intron. The potency of the compound was greatest when added at the time of infection (IC_{50} = 15 μM) with a 5- to lo-fold reduction in potency when the oligonucleotide was added 1 h post-infection. A 20% inhibition in splicing was observed in oligonucleotide treated infected cells *versus* untreated infected cells. Conjugation of the 12-mer oligomethylphosphonate with a psoralen-derivative increased the potency of the compound approximately 3-fold relative to the unconjugated compound. However, the psoralen conjugate required activation by UV irradiation following addition to the infected cells.

A study by Draper *et al.* (1990) using phosphodiester oligonucleotides complementary to two related region of the HSV-1 Vmw 65 mRNA, reported that an oligonucleotide targeted to the translation initiation region effectively inhibited HSV-1 replication. The other oligonucleotide was inactive, causing these authors to conclude that sequences within the same mRNA can exhibit differential sensitivities to antisense oligonucleotides.

Our laboratory has designed and tested several oligonucleotides which are complementary to the translation initiation regions of several mRNAs of HSV. Oligonucleotides which target the HSV UL13 mRNA were found to be effective inhibitors of HSV replication, as measured in an infectious yield assay (Draper & Brown-Driver, 1991; Draper *et al.,* submitted). The protein encoded by the UL13 gene has been putatively identified as a phosphotransferase which may be involved in the phosphorylation of viral capsid proteins (Smith *et al.,* 1986; Stevely *et al.,* 1985). Preliminary screening experiments revealed that phosphorothioate oligonucleotides were significantly more potent than phosphodiester and methylphosphonate oligonucleotides (Draper & Brown-Driver, 1991; Draper *et al.,* submitted). One of the most potent compounds evaluated was ISIS 1082, a 21-mer phosphorothioate oligonucleotide, targeted to a secondary initiation codon present in HSV-1 and HSV-2 UL13 mRNA. This compound inhibited both HSV-1 (KOS strain) and HSV-2 (HG52 strain) replication in an infectious yield assay. Site specific cleavage of synthetic UL13 transcripts was induced by addition of ISIS 1082 in RNA processing extracts of HeLa cells suggesting that ISIS 1082 may inhibit expression of the UL13 gene product by inducing RNAase H specific cleavage of UL13 mRNA.

Evaluation of the compound in infectious yield assays using acyclovir sensitive and resistant strains and in comparative dose responses with acyclovir and other phos-

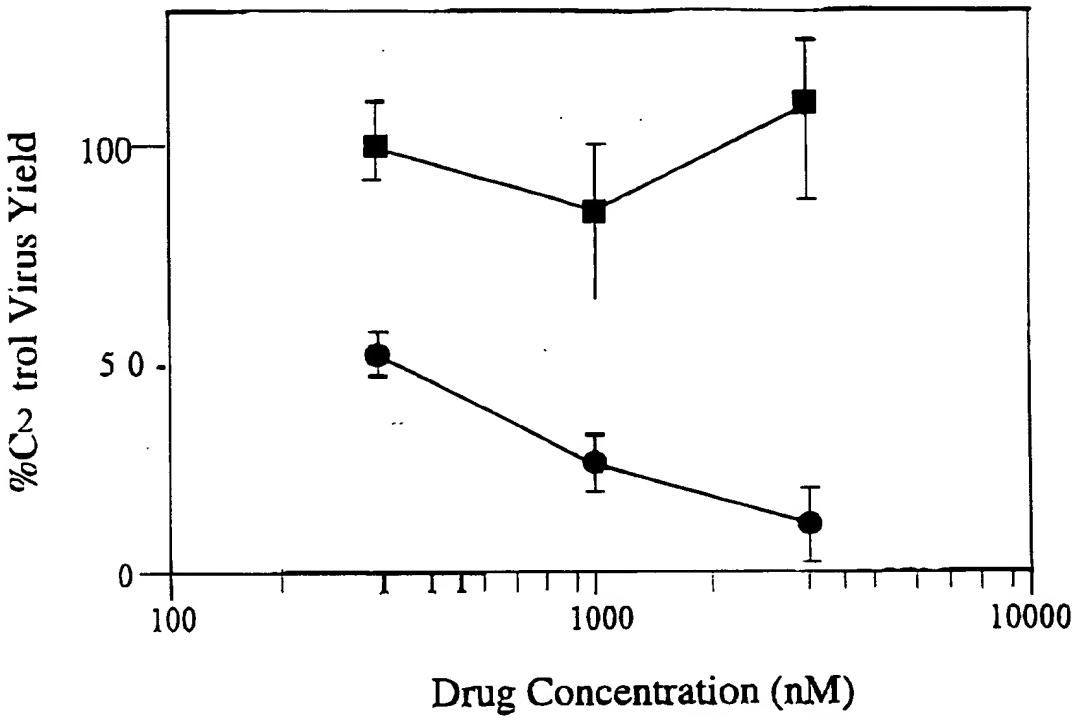

Figure 1 Sensitivity of an acyclovir resistant strain of HSV-1 (DM 2.1, thymidine kinase deletion mutant) to ISIS 1082 (●) and acyclovir (■). Activities were measured in an infectious yield assay and expressed as a percent of untreated infected cell virus yield

phorothioate oligonucleotides provided more evidence that ISIS 1082 produces its antiviral activity via a sequence specific antisense effect. First, ISIS 1082 inhibits the replication of the HSV-1 strain KOS in HeLa cells by 50% and 90% at concentrations of approximately 300 nM and 2 μM, respectively. ISIS 1082 was 3- to lo-fold more potent then a phosphorothioate oligonucleotide of similar length and equivalent (but scrambled) nucleotide base composition when tested against certain strains of HSV-1 and HSV-2. In addition, it was found that ISIS 1082 was active against a number of acyclovir resistant strains of HSV-1. Figure 1 shows the activity of ISIS 1082 against the thymidine kinase deletion mutant strain, DM2.1. Acyclovir did not inhibit the replication of this strain. However, treatment with ISIS 1082 resulted in a dose-dependent decrease in infectious viral yield that was equivalent to that observed against the KOS strain of HSV-1. At concentrations as high as 100 μM of ISIS 1082, only minimal effects on host cell growth and metabolism were observed (Crooke *et al.)*. This lack of *in vitro* toxicity is again consistent with the postulated highly selective mode of action of the antisense compound.

In vivo activities. Earlier preliminary reports have suggested *in vivo* activities of antisense drugs against HSV infections. One report indicated that an oligomethyl-phosphonate was active in a mouse model of herpes simplex virus 1 infection (Kulka *et al.*, 1989). Two additional laboratories have reported on the activity of phosphoro-thioates against HSV-1 infections in mouse models of ocular herpetic keratitis (Kimura *et al.*, 1991; Metcalf *et al.*, 1991).

Recent data have demonstrated that topical application of ISIS 1082 in an aqueous buffer to the cornea of mice infected with HSV-1 (KOS) resulted in curative activity at drug concentrations of 0.3% and 5% (Brandt *et al.*, 1991; Brandt *et al.* submitted). The activity of ISIS 1082 in this model was equivalent to trifluorothymidine and exhibited no local or systemic toxicities. ISIS 1082 is currently being studied in rabbit models of HSV-1 induced epithelial keratitis and other animal models of dermal and systemic HSV infection to better define the pharmacology of the compound.

Antisense oligonucleotides directed to intercellular adhesion molecules

To date most reports of antisense oligonucleotide activities in non-viral infection models have focused on oncogene targets and receptor signaling targets as seen in Table I. Our laboratory has recently explored the use of antisense oligonucleotides to pharmacologically manipulate the expression of certain cellular adhesion molecules (Chiang *et al.*, 1991).

Rationale for adhesion molecules as antisense targets

The binding of circulating leukocytes to vascular endothelium is an obligatory step in the emigration of leukocytes out of the vasculature to the site of infection or injury (Harlan, 1985). Several endothelial proteins have recently been identified which mediate the adherence of leukocytes to inflamed vascular endothelium and subsequent migration out of the vasculature (Stoolman, 1989; Osborn, 1990; Springer, 1990). One such protein, ICAM-1, is a 95-105 kD glycoprotein first identified by the ability of a monoclonal antibody to block phorbol ester induced aggregation of a B-cell line (Rothlein *et al.*, 1988). The cellular distribution of ICAM-1 is different from other endothelial cell adhesion molecules in that it is expressed in both endothelial cell and non-endothelial cells including leukocytes, fibroblasts, keratinocytes and other epithelial cells (Table II). ICAM-1 binds circulating leukocytes through LFA-1 (CD11a, CD18), a member of the β_2 integrin family (Marlin & Springer, 1987). ICAM-1 is a member of the immunoglobin gene superfamily containing five immunoglobulin domains (Simmons *et al.*, 1988; Staunton *et al.*, 1988; Tomassini *et al.*, 1989). Expression of ICAM-1 is inducible by a number of cytokines including IL-1, TNF-α and IFN-γ (Rothlein *et al.*, 1988; Stoolman, 1989; Osborn, 1990; Springer, 1990). The broad tissue distribution of ICAM-1 suggests that it is not only involved in the emigration of leukocytes out of the vasculature, but may play a more extensive role in immune responses. Additional roles suggested for ICAM-1 include localization of leukocytes to the area of inflammation in extravascular spaces, enhancement of the recognition of antigen presenting cells by T lymphocytes, formation of lymphocyte germinal centers, enhancement of natural killer cell response and differentiation of thymocytes (Rothlein *et al.*, 1986; Dustin *et al.*, 1986 & 1988; Makgoba *et al.*, 1988; Altmann *et al.*, 1989; Boyd, 1989; Robertson *et al.*, 1990; Springer, 1990). In addition ICAM-1 is the receptor for over 90% of the rhinovirus serotypes (Staunton *et al.*, 1989; Tomassini *et al.*, 1989).

***In vitro* inhibition of ICAM-1 expression by antisense oligonucleotides**

During the initial evaluation of a series of phosphorothioate oligonucleotides targeted to specific sites within the ICAM-1 mRNA it was found that the cationic lipid,

Table II. Leukocyte adhesion molecules

Endothelial CAM	*Expressed on other cells*	*Gene family*	*Induction kinetics*	*Leukocyte ligand*	*Type of leukocyte bound*
ICAM-1	Keratinocytes, fibroblasts, leukocytes, etc.	Immunoglobulin	4 h to 72 h	LFA-1, MAC-1	Lymphocytes, monocytes, granulocytes
ICAM-2	Activated lymphocytes	Immunoglobulin	Constitutively	LFA-1	Lymphocytes, monocytes, granulocytes
VCAM-1	No	Immunoglobulin	4 h to 72 h	VLA4	Lymphocytes, monocytes
ELAM-1	No	LEC-CAM	2 h to 18 h	Carbohydrate	Granulocytes monocytes, memory T cells
GMP-140	Platelets	LEC-CAM	5 min to 2 h	Carbohydrate	Granulocytes, monocytes

DOTMA markedly enhanced the activity of the antisense oligonucleotides used in this study. DOTMA was originally described as a vehicle for transfection of DNA into cells (Felgner *et al.*, 1987). Cationic lipid delivery methods differ from normal liposomal delivery methods, in that the DNA or oligonucleotide is not encapsulated within the liposome, but rather is associated with the surface of the liposome through ionic interactions. Preliminary data in certain cell lines indicate that DOTMA enhances cell association of oligonucleotides at least lo-fold and markedly changes the intracellular distribution of the oligonucleotide, with apparently less oligonucleotide being concentrated in endosomes or lysosomes and more found in the nucleus (Chiang *et al.*, 1991; Bennett *et al.*, in preparation). Therefore, in some cells DOTMA will enhance oligonucleotide entry into the cytoplasm of cells similar to direct microinjection. The use of DOTMA has the advantage over microinjection experiments in that oligonucleotides can be introduced into large number of cells allowing biochemical analysis to be performed. In addition, it was determined that DOTMA had no effect on the expression of ICAM-1 when used at concentrations that maximized oligonucleotide uptake and activity (Chiang *et al.*, 1991). The use of DOTMA in these experiments allowed us to determine which regions on the ICAM-1 mRNA serve as the best target sites for antisense oligonucleotides and determined the mechanism by which antisense oligonucleotides inhibit ICAM-1 expression. To our knowledge this is the first report demonstrating that cationic lipids enhance antisense oligonucleotide activity in mammalian cells.

Using DOTMA as a formulation medium we have demonstrated that antisense oligonucleotides which target human ICAM-1 mRNA inhibit the expression of ICAM-1 in two cell culture systems HUVEC and a human lung carcinoma, A549 (Chiang *et al.*, 1991). Screening antisense oligonucleotides which target a number of sites on the ICAM-1 mRNA revealed that two sites were especially sensitive to inhibition with antisense oligonucleotides; the AUG translation initiation codon and specific sequences in the 3'-untranslated region. Data from these studies suggest that hybridization affinity is important for antisense oligonucleotides, as truncated versions of active oligonucleotides (<20-mers) exhibit decreased activity, however, hybridization affinity is not sufficient to ensure antisense activity. Therefore, target site selection is also an important parameter to consider when designing antisense oligonucleotides.

The most active ICAM-1 antisense oligonucleotide targets the 3'-untranslated region of the ICAM-1 mRNA. ISIS 1939 hybridizes to the ICAM-1 mRNA, nearly 300 bases 3'- to the translation termination site, therefore it should not directly affect translation of the protein. This oligonucleotide was shown to inhibit the expression of ICAM-1 in endothelial cells as measured by ELISA using a monoclonal antibody to ICAM-1 (Figure 2). Under equivalent experimental conditions treatment of endothelial cells with ISIS 1939 blocked the adhesion of HL60 cells. Thus the blockade of ICAM-1 expression was coincident with the loss of functional activity of the protein. Oligonucleotides which hybridized to other sequences in the 3'-untranslated region of ICAM-1 mRNA were not as effective as ISIS 1939 (Figure 2). Therefore, the effects of ISIS 1939 are unique to the target site to which it hybridizes.

ICAM-1 mRNA contains three repeats of a consensus sequence, AUUUA, thought to be involved in destabilization of mRNA (Caput *et al.*, 1986; Shaw & Kamen, 1986; Brawerman, 1989). An oligonucleotide that targets those sequences was shown to exhibit weak activity. However, ISIS 1939 targets an area approximately 200 bases 5'- to the AUUUA sequences. The region targeted by ISIS 1939 is predicted to be a stable stem loop structure which when bound would disrupt the structure. Analysis of steady state mRNA levels from oligonucleotide treated cells revealed that ISIS 1939

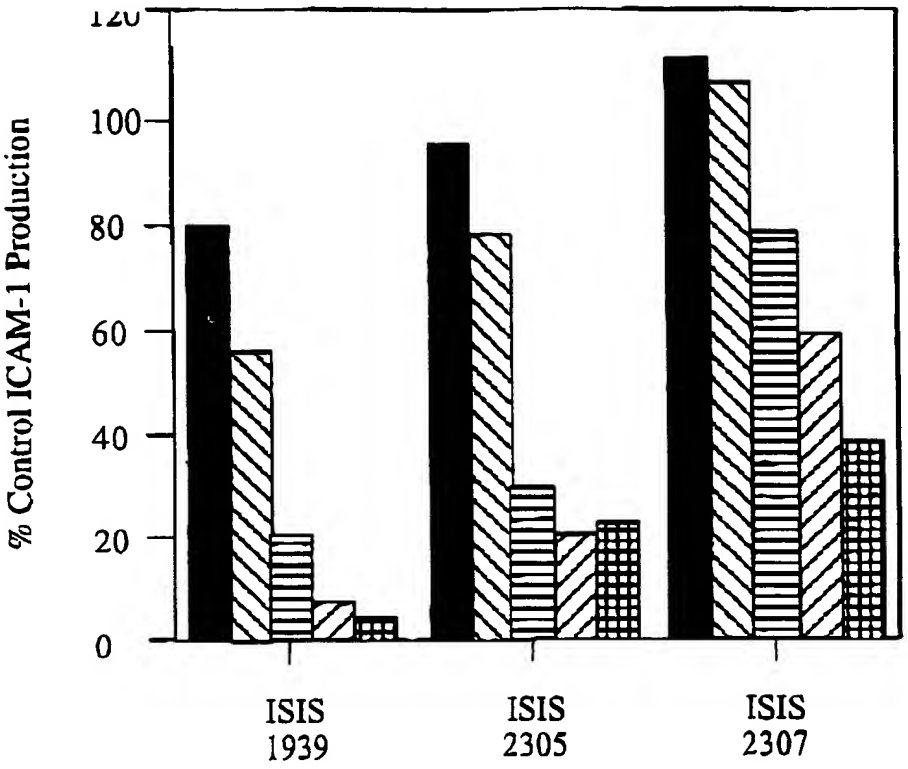

Figure2 Inhibition of IL-1 induced ICAM-1 expression in AS49 cells with antisense oligonucleotides which hybridize to the 3'-untranslated region of ICAM-1 mRNA. Cells were treated with phosphorothioate oligonucleotides (20 mers) at concentrations of ■ 0.1 μM ▢ 0.3 μM ▢ 0.5 μM ▢ 0.7 μM ▩ 1.0 μM in the presence of DOTMA. ICAM-1 expression was measured by ELISA using an ICAM-1 monoclonal antibody 84H10

specifically reduced the quantity of ICAM-1 mRNA per cell. The reduction of ICAM-1 mRNA was not due to decreased transcription of the ICAM-1 gene as analysed by nuclear run-off reactions. Therefore, ISIS 1939 must destabilize the ICAM-1 mRNA either by an RNase H dependent mechanism and/or by modulating natural processes which help to stabilize the ICAM-1 mRNA.

Oligonucleotides targeted to certain other specific sites within ICAM-1 mRNA were found to be potent inhibitors of ICAM-1 protein expression and cell adhesion. These oligonucleotides were targeted to sequences within the 5' untranslated region and the translation initiation region. The oligonucleotide targeted to the translation initiation region did not cause a reduction in the steady state level of ICAM-1 mRNA; unlike that found with ISIS 1939. Taken together these data suggest that different oligonucleotides targeted to different sites on an RNA may inhibit the production of a protein by different mechanisms.

Summary

The notion of using antisense oligonucleotides as pharmacologic agents is a derivative of the central dogma of molecular biology and knowledge of the physical and

chemical properties that govern the structure of nucleic acids. The practical evidence that antisense oligonucleotides can be drugs is a result of the work of a number of laboratories, including those cited in this review.

Key to the continued progress in the field of antisense therapeutics is the realization that oligonucleotides and their RNA targets work via the same principles of pharmacology that govern the actions of all other classes of drugs. Considering the properties of drugs that define their pharmacologic value, such as ligand-receptor binding affinity and fidelity and realizing the intrinsic properties of oligonucleotides, it is very clear that these compounds have enormous potential value in treating human diseases.

During the next few years a number of oligonucleotide compounds will enter into clinical trials. These first generation antisense drugs (e.g. phosphorothioates) will encounter many of the same issues and hurdles that confront all novel pharmaceutical agents; large-scale process development, adequate methods and tools to define clinical pharmacokinetics and metabolism, etc. Another important component of this process is the continued examination and definition of the molecular pharmacodynamics and pharmacokinetics of these drugs. We need to better understand how the structure and function of RNA defines the sensitivity of specific target sites to antisense oligonucleotides, the precise role of RNase H and other intracellular enzymes and proteins in the mechanism of action in oligonucleotides, the process by which oligonucleotides penetrate cellular membranes and distribute in cells, the non-sequence specific interactions that oligonucleotides can engage in both in and out of cells, and the metabolic pathways (both nuclease and non-nuclease) and metabolites that are likely to play a role in the metabolism of antisense drugs. The combination of this molecular, cellular, and clinical information will allow us to better determine the specific molecular targets and diseases that can be successfully treated with the first generation of antisense drugs. As important, it will define the biology, chemistry, and pharmacology of second and third generation antisense drugs.

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THERAPEUTIC APPLICATIONS OF OLIGONUCLEOTIDES

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INTRODUCTION

First proposed in 1978 by Zamencik & Stephenson (1), oligonucleotide therapeutics represent a new paradigm for drug discovery. The technology focuses on a class of chemicals, oligonucleotides, that has not been studied as potential drugs before and employs them to intervene in biological processes that likewise have not been studied previously as sites at which drugs might act.

The paradigm has resulted in substantial enthusiasm because oligonucleotides may display dramatic increases in affinity and selectivity for their nucleic acid targets compared to traditional drugs. Furthermore, antisense technology may facilitate rational drug design. Table 1 compares affinities and the potential for selectivity of oligonucleotides versus traditional drugs. The comparison is based on average affinities of typical traditional drugs in optimized assays with purified receptors and data derived from a 21-mer phosphorothioate oligonucleotide in binding assays performed in 1M NaCl. Hybridization varies substantially as a function of ionic strength, and the affinities at 100 mm NaCl in the presence of Mg^{2+} for the 21-mer are significantly lower. Furthermore, affinities may be lower in physiological systems with RNA that has secondary structure, so these comparisons present the opportunity in its broadest dimensions.

A number of terms have been coined and often misused to describe various

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Table 1 Affinity and selectivity of traditional and oligonucleotide drugs. Affinity constants were determined as described in text

Traditional drug		Oligonucleotide drug (1M NaCl)	
Affinity for receptor	10^9	Affinity for receptor sequence	10^{19}
Affinity for isotype	10^6 –10^8	Affinity for one base mismatch	10^{16}
Maximum affinity for other proteins	10^6 –10^9	Maximum affinity for nucleic acid binding proteins	10^{12}–10^{13}
ΔKd	1–10^3	Δ kD	10^4–10^{11}

components of the overall approach to using oligonucleotides as therapeutic agents. *Antisense* describes the interaction between oligonucleotides complementary to (sense) pre-mRNA[1] or mRNA molecules; these inhibit the oligonucleotides production of the protein product. The term has been broadened to describe any therapeutic oligonucleotide interaction with nucleic acids. *Triplex* denotes the interaction between oligonucleotides and double-stranded DNA that may result in inhibition of transcription. RNA structures display double-stranded regions, however, and thus the formation of triple-stranded structures in RNA is also possible. *Aptamers* describes the use of oligonucleotides to bind to nucleic acid binding proteins.

SCOPE AND OBJECTIVES OF THE REVIEW

This review focuses strictly on the use of oligonucleotides designed to interact with nucleic acids as therapeutics. The objectives are to place oligonucleotide therapeutics in the context of modern drug discovery and development and to summarize recent progress.

[1]Abbreviations: BFGF, basic fibroblast growth factor; cAMP, cyclic AMP; CH_3-P, methylphosphonate oligonucleotides; EGF, epidermal growth factor; ELAM, endothelial cell adhesion molecule; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HD, hepatitis B; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-cell lymphotrophic virus; ICAM, intracellular adhesion molecule; IGF, insulin-like growth factor; IL-1, interleukin 1; introns, intervening sequences; IV, influenza virus; mRNA, messenger ribonucleic acid; P, phosphodiester oligonucleotides; P-acridine, phosphodiester oligonucleotide conjugated with acridine moiety; PCNA, proliferating cell nuclear antigen; PLA_2, phospholipase A_2; P-lipid, phosphodiester oligonucleotide conjugated with lipid moiety; PMA, phorbol mysteric acid; P-S, phosphorothioate oligonucleotides; Rev, regulation of virion protein expression; RSV, Rous sarcoma virus; TAR, Tat response element; TBE, tick-borne encephalitis; Tm, thermal transition point; VSV, vesicular stomatitis.

BASIC CONSIDERATIONS

Conceptually, oligonucleotide drug effects can be rationalized by traditional receptor theory and basic concepts concerning drug action. Within the broad context of pharmacological theory, however, a number of differences influence rational drug design and the potential utility of these agents.

Pharmacodynamics

RNA INTERMEDIARY METABOLISM Oligonucleotides are designed to modulate the information transfer from the gene to protein—in essence, to alter the intermediary metabolism of RNA. Figure 1 summarizes these processes.

RNA intermediary metabolism is initiated with transcription. The transcription initiation complex contains proteins that recognize specific DNA sequences and locally denature double-stranded DNA, thus allowing a member of the RNA polymerase family to transcribe one strand of the DNA (the antisense strand) into a sense pre-mRNA molecule. Usually during transcription, the 5' end of the pre-mRNA is capped by adding a methyl-guanosine and most often by methylation of one or two adjacent sugar residues. This enhances the stability of the pre-mRNA and may play a role in a number of key RNA processing events (2). Between the 5' cap and the site at which translation is initiated is usually a stretch of nucleotides; this area may play a key role in regulating mRNA half-life (3).

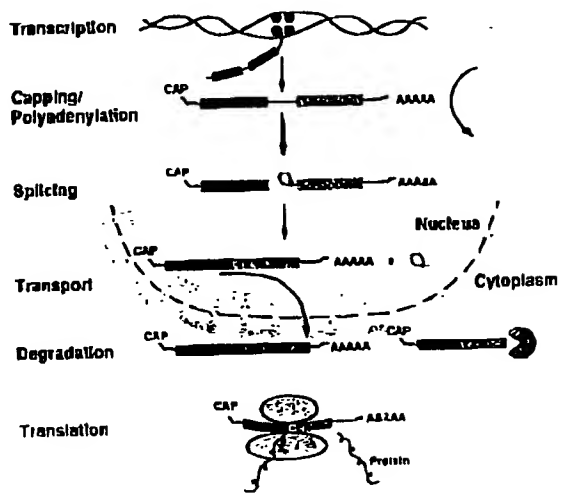


Figure 1 RNA processing.

Similarly, the 3' end of the pre-mRNA usually has a stretch of several hundred nucleotides beyond the translation termination signal. This area often plays an important role in determining mRNA half-life. Moreover, post transcriptionally, most pre-mRNA species are polyadenylated. Polyadenylation stabilizes the RNA, is important in transport of mature mRNA out of the nucleus, and may play important roles in the cytoplasm as well (4, 5).

Because eukaryotic genes usually contain intervening sequences (introns), most pre-mRNA species must have these sequences excised and the mature RNA spliced together. Splicing reactions are complex, highly regulated, and involve specific sequences, small molecular weight RNA species, and numerous proteins. Alternative splicing processes are often used to produce different mature mRNAs and, thus, different proteins. Even though introns are often considered waste, important sequences are conserved, and some introns may play a variety of regulatory roles.

Mature mRNA is exported to the cytoplasm and engages in translation. mRNA half-life varies from a few minutes to many hours and appears to be highly regulated (3).

Each step shown in the pathway is a composite of numerous steps, and each step is theoretically amenable to intervention with oligonucleotides. For virtually no mRNA is the pathway fully defined, however, and available information is insufficient to determine the rate-limiting steps in the intermediary metabolism of any mRNA species (6, 7).

AFFINITY The affinity of oligonucleotides for their receptor sequences results from hybridization interactions. The two major contributors to the free energy of binding are hydrogen bonding (usually Watson-Crick base pairing) and base stacking in the double helix that is formed. As mentioned, affinity is affected by ionic strength. Affinity results from hydrogen bonding between complementary base pairs; the reduction in entropy results from the stacking of the coplanar bases. Consequently, affinity increases as the length of the oligonucleotide receptor complex increases. Affinity also varies as a function of the sequence in the duplex. Nearest neighbor rules allow the prediction of the free energy of binding for DNA-DNA and RNA-RNA hybrids with relatively high precision (8, 9). Less information is available to develop predictions for DNA-RNA duplexes. A common misconception is that DNA-RNA duplexes are more stable than DNA-DNA duplexes. In fact, the relative stability of these duplexes varies as a function of the sequence. RNA-RNA duplexes are typically the most stable (S. M. Freier, unpublished results).

As with other drug-receptor interactions, activity requires a minimum level of affinity. For many targets and types of oligonucleotides, the minimum length of an oligonucleotide may be 12–14 nucleotides.

SPECIFICITY Specificity derives from the selectivity of Watson-Crick or other types of base pairing. The decrease in affinity associated with a mismatched base pair varies as a function of the specific mismatch, the position of the mismatch in a region of complementarity, and the sequence surrounding the mismatch. As an example, Table 2 compares the impact of various mismatches centered in two complementary 18-mers. The $\Delta\Delta G^{\circ}_{37}$ or change in Gibbs free energy of binding induced by a single mismatch varies from $^{+}0.2$ to $^{+}4.9$ kcal/mol per-modification at 100 mM NaCl. Thus, a single base mismatch results in a change in affinity of approximately 500-fold (10). Modifications of oligonucleotides may alter specificity. In fact, we have synthesized modified bases with substantially enhanced selectivity and others that display virtually no preferences for different bases.

Table 2 Effects of single-base mismatches on duplex stability. Absorbance vs temperature profiles were measured at 4 μM each strand in 100 mM Na^{+}, 10 mM phosphate, 0.1 mM, EDTA, pH 7.0.

X strand: 5'-d(CTC GTA CCx TTC CGG TCC)-3' fully phosphorothioate
X = dA, dC, dG, or T

Y strand: 5'-(GGA CCG GAA yGG TAC GAG)-3' fully RNA
y = A, C, G, or U

X	Y	T_m[1] (°C)	ΔT_m (°C)	ΔG°_{37} (kcal/mol)	$\Delta\Delta G^{\circ}_{37}$ (kcal/mol)
dT	rA	53.6	—	−11.6	—
dT	rC	40.8	−12.8	−8.9	+2.6
dT	rG	50.0	−3.6	−10.5	+1.1
dT	rU	41.9	−11.7	−9.1	+2.5
dG	rC	56.9	—	−13.1	—
dG	rA	42.3	−14.6	−8.9	+4.2
dG	rG	45.0	−11.8	−9.3	+3.8
dG	rU	45.7	−11.1	−9.8	+3.3
dC	rG	59.0	—	−12.5	—
dC	rA	43.3	−15.7	−9.3	+3.2
dC	rC	39.5	−19.4	−8.7	+3.9
dC	rU	40.0	−19.0	−8.7	+3.8
dA	rU	52.7	—	−11.4	—
dA	rA	42.7	−10.0	−9.3	+2.2
dA	rC	42.7	−10.0	−9.1	+2.3
dA	rG	44.5	−8.1	−9.3	+2.1

[1] T_ms and free energies of duplex formation were obtained from fits of the absorbance vs. temperature data to a two-state model with linear sloping baselines. Reported parameters are averages of at least three experiments.

Based on the differences in affinity of oligonucleotides for their complementary target sequence, calculations suggest that unmodified oligodeoxynucleotides between 11–15 in length should be able to bind selectively to a single RNA species in the cell (11). Studies in our laboratories have demonstrated that affinities predicted by nearest neighbor analyses are highly useful in rational drug design (10). For example, by using strategies based on nearest neighbor predictions, oligonucleotides can be designed that selectively inhibit the production of mutant RAS containing a single base change in the mRNA vs. normal RAS in cells in tissue culture (B. P. Monia et al, manuscript submitted).

NUCLEIC ACID SELECTIVITY The 2'-hydroxyl in RNA results in the sugar assuming a different conformation from that in DNA. RNA-RNA duplexes assume an A-form double helix whereas DNA-DNA duplexes assume a B-form double helix. Consequently, oligonucleotides can be modified to bind more tightly to RNA or DNA sequences. For example, Table 2 shows the effect of 2'-modifications at every position of a 15-mer on T_m and ΔG^0_{37} for DNA and RNA targets and demonstrates that 2'-O-methyl substitutions increase T_m for RNA by 1.4° per modification compared to DNA (12).

RNA STRUCTURE RNA can assume a variety of secondary structures deriving from intramolecular base pairing. The simplest structures are stem-loops in which double-stranded regions are interspersed with loops and random coils. More complex structures described as *pseudoknots* also form (13). These structures are profoundly important in determining RNA function and influencing the ability of oligonucleotides to bind to their RNA targets. The types of effects of bound oligonucleotides on RNA function are affected by RNA structures as well.

Pharmacokinetics

As with any other class of drugs, oligonucleotide drugs must attain a sufficient concentration at their receptor for a sufficient period to display activity. Inasmuch as most of the targets for oligonucleotides are intracellular, oligonucleotides must be relatively stable in and outside the cell and must be able to traverse the cellular membrane.

NUCLEASE STABILITY Oligonucleotides may be degraded by nucleases. Nucleases that degrade DNA or RNA from either the 5' or 3' terminus are known as *exonucleases;* those that cleave internally are *endonucleases*. Numerous nucleases exist and have been shown to degrade oligonucleotides. Although in serum the dominant nuclease activity is 3' exonuclease, in cells and other bodily fluids 3' and 5' exonucleases and endonucleases are present.

In serum, phosphodiester oligodeoxynucleotides are rapidly degraded. The rate of degradation varies as function of the sequence and length of the oligonucleotide and the type of serum (14–16). Typically, half-lives of phosphodiester oligodeoxynucleotides range from 15 to 60 min in most sera. Heat inactivation of serum reduces the rate of degradation of oligonucleotides. Oligoribonucleotides are significantly less stable than oligodeoxynucleotides.

Work from many laboratories has demonstrated that a wide range of modifications may be used to enhance the stability of oligonucleotides. Phosphate modifications have been shown to result in marked increases in stability (see Table 3). Phosphorothioate oligonucleotides have been shown to be extremely stable in media, cells and cell extracts, serum, various tissues, urine and stable to most nucleases (16–20). The half-life of phosphorothioate oligonucleotides is greater than 24 hr in nearly all environments tested. Furthermore, phosphorothioates have been shown to be stable to various restriction endonucleases when in duplexes. In general, one of the diastereomers is cleaved slowly and the other is entirely resistant (21–24).

The non-ionic methylphosphonate analogs have also been shown to be extremely stable to nucleases (25–31). Again, these oligonucleotides are diasteresomeric at each modified phosphate, and the R isomer is slightly more sensitive than the S isomer to degradation by nucleases (16, 32).

Table 3 Effects of 2' modifications on hybridization and stability. Duplex hybridization was evaluated from absorbance vs. temperature profiles at 260 nm in 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7.0 at 8 μM strand concentration.

		Hybridization		Serum stability
Modification[1]	Positions	T_m[2] vs DNA (°C)	T_m vs RNA (°C)	$T_{1/2}$
Phosphodiester	All	50.5	39.0	1 h
Phosphorothioate	All	43.2		>24 h
2'-O-nonyl dA	12 13 14		41.3	60 h
2'-O-allyl	12 13 14	50.3	40.8	10 h
2'-O-benzyl	12 13 14	45.5	37.8	18 h
2'-O-aminopropyl	12 13 14	53.7	42.0	1 h
2'-fluoro P + S	2'F in 12 13 14 P = S in all	47.2	36.5	>>24 h

[1] 15 mer: CGA CTA TGC AAA AAC

[2] T_m is the temperature at which half the strands are in the duplex state and half are in the coil state. T_m was obtained from a nonlinear least squares fit of the experimental data to a modified two-state model with linear sloping baselines (224)

Other classes of modifications that have been reported to result in substantial nuclease stability include the phosphoramidates (19, 33) and isopropyl phosphate triesters (34, 35). Interestingly, ethylphosphate triesters were shown to be cleaved after being deethylated in cells (27, 36). Oligonucleotides containing α-anomers in the sugar moiety are substantially more stable in serum and cells than natural phosphodiesters (14–15, 37–41).

Modifications at the 2'-position of the sugar have also been shown to enhance nuclease stability (42). 2'-O-methyl-oligonucleotides were shown to be significantly more resistant than unmodified oligonucleotides, and 2'-O-allyl modified oligonucleotides were even more stable (43). In studies in our laboratory, a large number of 2'-modifications have been characterized. 2'-O-methyl analogs were highly resistant to nucleases in serum and cells. Modifications as bulky as nonyl groups were shown to have only a minor negative effect on hybridization and to impart high levels of nuclease stability In contrast, 2'-fluoro derivatives were nearly as sensitive to nucleases as unmodified oligoribonucleotides (12, 44). Table 3 provides a comparison of affinities to RNA and stabilities in serum for several 2' modified oligonucleotides (45). Although numerous other modifications have been studied, either insufficient data concerning hybridization properties or nuclease stabilities have been reported to support conclusions or their hybridization properties were unattractive. For example, open ring sugar analogs of adenosine were reported to be nuclease stable (46). Acyclic pentofuranosyl modified oligonucleotides were reported to be nuclease resistant, but the T_m for these oligonucleotides was reduced 9–15 degrees per modification (47). Other acyclic sugars have been reported but, again, the hybridization properties were poor (48). Carbocyclic modified oligothymidylates were reported to be nuclease resistant and to hybridize to oligodeoxyadenosine with higher affinity than natural oligodeoxy-thymidylate (49, 50), but studies on mixed sequences have not been reported.

A wide variety of phosphate replacements have also been studied. In earlier work, the phosphodiester was replaced with esters, amides, and various polymeric materials, but these modifications were not designed to be used as antisense oligonucleotides and, therefore, are largely unattractive (12, 16). More recently, formacetal replacement of the phosphate has been reported by two groups to result in oligonucleotides with acceptable hybridization properties and nuclease resistance (51–53).

Other modifications for which little information is available include sulfonamide replacement of phosphate (54), diphosphonate dinucleotides (55), acetamide linkages (56, 57), and phosphonyl methyl linkages (58). These and other modifications are discussed in detail in two recent reviews (12, 16). In our laboratories, a number of other novel backbone modifications have been synthesized. Given the number of novel synthetic approaches and molecules

and the number of laboratories now involved, a substantial increase in the repertoire of backbone-modified oligonucleotides with desirable properties is likely in the near future.

In addition to uniform modifications, a number of pendant groups at the 5' and/or 3' termini and more recently in internal positions of oligonucleotides have been reported to enhance nuclease stability. Modifications include intercalating agents (59–62) and poly-L-lysine (63, 64) at the 5' or 3' terminus and a number of modifications such as amino-alkoxy (65), anthraquinone (66), and alkyl groups (45). Moreover, heterocycle modifications, including pendant groups from the N2 site of guanine (67, 68), pendant groups from 3-deazaguanine (69), and 5- and 6-position modifications of deoxycytidine and thymidine (70), have shown increased stability to nucleases of varying levels.

In conclusion, numerous medicinal chemical strategies can be employed to create oligonucleotides with varying degrees of nuclease stability. The choice of the modification(s) employed is dictated by the level of stability desired and other desired properties of the oligonucleotides. It is now possible to design oligonucleotides that display excellent hybridization characteristics and half-lives, that range from minutes to several days when oligonucleotides are incubated with nucleases, serum, cells, or cell extracts.

INTRACELLULAR STABILITY Although considerable confusion and controversy exist with regard to the stability of oligonucleotides in cells in tissue culture and the ability to predict intracellular stability of oligonucleotides based on stability in sera, a consensus opinion is emerging. The nuclease activity of sera derived from different species varies. Fetal calf serum is more active than mouse serum, and human serum appears to have the least nuclease activity (G. D. Hoke, unpublished observations). All sera display substantial nuclease activity, however, and there are significant lot-to-lot variations. In all sera tested, 3' exonucleases constitute the primary nuclease activity (12, 16, 71). In a number of publications, fetal calf serum used in tissue culture experiments has been heated to inactivate nucleases. Again, however, conditions were not standardized, and in some lots of sera, heating to 65°C for 30 min does not inactivate all nucleases (16).

Another factor that has contributed to confusion is that a variety of labeling methods and analytical techniques have been employed. Studies have employed $3'^{32}P$ and $5'^{32}P$ labeled oligonucleotides, $5'^{35}S$ labeled oligonucleotides, and oligonucleotides labeled with fluorescent pendant groups at the 5' terminus (14–16, 72). Relatively few studies have used uniformly labeled oligonucleotides. Furthermore, relatively few studies have rigorously separated intact oligonucleotides from degradation products, and even fewer have performed careful kinetic studies.

Studies in our laboratory have employed either phosphodiester oligonucleotides uniformly labeled with ^{32}P or phosphorothioate oligonucleotides uniformly labeled with ^{35}S. The kinetics of degradation have been studied with several cell lines in vitro and cytoplasmic and nuclear extracts derived from HeLa cells. In contrast to a number of studies, in all cells studied to date, phosphodiester oligonucleotides were degraded within 15–30 min of incubation (71, 73). In contrast, phosphorothioate oligonucleotides of 15, 21, and 30 nucleotides in length and various sequences were stable for at least 24 hr when incubated with various cells. In studies in HeLa cells in which ISIS 1082, a 21-mer phosphorothioate, was incubated with the cells, then extracted from cells at various time points and analyzed on polyacrylamide gels, the compound was intact for four days (73).

Methylphosphonate oligonucleotides have also been shown to be stable in a variety of cells lines and extracts (14). No other class of oligonucleotides, however, has been sufficiently studied to allow definitive conclusions.

CELLULAR UPTAKE AND DISTRIBUTION Antisense oligonucleotides typically are 15–30 nucleotides long and thus have molecular weights that range from 4500–9000 daltons. The charge carried by phosphodiesters is, of course, negative and they are highly water soluble. The charge and hydrophilicity of modified oligonucleotides vary depending on the modifications. Consequently, membrane transport and cellular distribution are likely to vary widely as a function of the modifications introduced into oligonucleotides. For the two classes of modified oligonucleotides for which significant data have been reported—methylphosphonates and phosphorothioates—this is clearly the case. For both classes of oligonucleotides, the evidence is compelling that they do enter many cells at pharmacologically relevant concentrations.

Methylphosphonates are uncharged and lipophilic. Although thought to be taken up by most cells in tissue culture via passive diffusion, detailed studies of the kinetics of cellular uptake, distribution, and metabolism of uniformly labeled methylphosphonates have not been reported. Studies in Syrian hamster fibroblasts on oligonucleotides 3–9 nucleotides in length showed linear cell association for 1 hr, then reduced uptake. At equilibrium, the intracellular concentration of oligonucleotide was reported to be equivalent to the extracellular concentration (27, 74). In another study, a 21-mer methylphosphonate labeled with ^{32}P at the 5' terminus was reported to be taken up by CV-1 cells. Cell association was linear for 2 hr. Unfortunately, however, studies proving that the cell-associated radioactivity represented intact oligonucleotide were not presented. Nor were detailed studies on characteristics of uptake or intracellular distribution presented (75).

Phosphorothioates are negatively charged, but because of the sulfur atoms they may be slightly more lipophilic than phosphodiesters and tend to bind nonspecifically to serum proteins. Studies in our laboratories have shown that phosphorothioate oligonucleotides bind to serum albumin and that in the presence of serum albumin, cell-association is reduced (73; G. D. Hoke et al, unpublished observations).

Studies employing a 28-mer phosphorothioate deoxycytidine that was uniformly labeled with ^{35}S demonstrated that when HeLa cells were incubated with 1 μM of the drug, significant intracellular concentrations were achieved. Cellular uptake was linear, reaching a plateau of 60 p mole/10^6 cells in 6 hr. Adsorption to the cell membrane was minimal. Uptake was also concentration-dependent, reaching a plateau at approximately 1 μM. The drug associated with HeLa cells was intact for 24 hr and was located in both nuclei and cytoplasm. Infection with herpes simplex virus type 2, but not type 1, increased cellular uptake (76).

Studies in our laboratories have confirmed and extended the observations on phosphorothioate oligonucleotides. The cellular uptake, distribution, and metabolism of ISIS 1082, a uniformly ^{35}S labeled 21-mer phosphorothioate with a mixed antisense sequence, have been characterized in HeLa cells and HeLa S_3 cells, a variant line conditioned to growth in suspension. Incubation of HeLa cells with 5 μM of the drug resulted in approximately 8% of input radioactivity being associated with the cells. Cell association was linear for approximately 8 hr, and approximately 20% of the cell-associated radioactivity appeared to be adsorbed to the membrane. Uptake was temperature-dependent, required viable cells, and was inhibited by metabolic poisons. Uptake was concentration-dependent, and was linear to 10 μM. Uptake was influenced slightly by calcium and magnesium and was saturable. Natural oligonucleotides and methylphosphonates did not compete for uptake while other phosphorothioates competed; however, different length and sequence phosphorothioates competed differently (73, 77).

We have also studied other phosphorothioates of various lengths and other cell lines. HL 60 cells appear to take up less phosphorothiate oligonucleotides than HeLa cells and HeLa S_3 cells take up very little drug (73). Although not directly compared, human umbilical vein endothelial cells also appear to take up less drug than HeLa cells. Thus, there is considerable variation in the extent of uptake as a function of cell type.

In all cells studied, and with all uniformly labeled phosphorothioate oligonucleotides of varying size and sequences, we have shown that these drugs are stable in cells and cytoplasmic and nuclear extracts. In HeLa cells, no degradation of intracellular ISIS 1082 was observed for four days (73). Preliminary studies confirmed that these oligonucleotides distributed to both cytoplasm and nuclei and showed that there is an active temperature-dependent efflux process as well (77, 78).

When incubated with cells in the absence of serum or heat-inactivated serum, several laboratories have reported the apparent uptake of phosphodiester deoxyoligonucleotides. Moreover, a number of laboratories have reported activities for phosphodiester oligonucleotides that apparently were due to cellular uptake and intracellular activities. The studies on cellular uptake are not fully convincing, however Loke et al (79) studied deoxythymidine oligonucleotides ranging from 3–20 nucleotides in length and labeled with acridine at the 3' terminus. They incubated HL60 and three other hematopoietic cell lines with 12.5 μM of the acridine labeled drug and used flow-cytometric analyses of acridine fluorescence to quantitate cellular uptake. Uptake was reported to decrease as the length of the oligonucleotide increased and to vary as a function of the cell type. Uptake achieved a plateau in HL60 cells in 50 hr and was inhibited by polynucleotides of any length. The authors concluded that the oligonucleotides were taken up by an endocytotic mechanism. Unfortunately, the stability of the oligonucleotide-acridine conjugate was not rigorously documented. Nor were possible effects of acridine in the uptake of the oligonucleotide rigorously explored. Additionally, possible quenching or enhancement of the fluorescence of acridine by cellular interactions was not explored. Finally, extrapolations from homopolymers to mixed sequences have not been proven to be valid.

Another study employing phosphodiester oligonucleotides reached similar conclusions (80). Again, for most of the experiments, oligodeoxythymidines of 8 to 16 nucleotides in length were incubated with L929 mouse fibroblasts in the absence of serum. Maximal uptake occurred within 2 hr and upon incubation with fresh medium, cell-associated ^{32}P was released. Substantial degradation of the 5' labeled oligonucleotide was observed within 2 hr, and the authors concluded that approximately 20% of the radioactivity was in nuclei. Again, the authors concluded that the most likely mechanism of uptake was endocytosis (80).

Other pendant modifications of phosphodiester oligonucleotides have also been studied. A 9-mer labeled with acridine at the 3' terminus was reported to be taken up by *Trypanosome brucei* (61). More recently, the same group has reported that a 9-mer coupled at the 3' terminus to acridine via a dodecanal linker was more active in cells expressing mutated RAS than a 9-mer with a 3' acridine only (81). 3' poly-L-lysine-oligonucleotides have been reported to be stable to serum nucleases and to have enhanced activity as compared to phosphodiesters. Uptake was not studied, however, (63, 82, 83). In a later publication, the uptake of a poly-L-lysine oligonucleotide conjugate was enhanced compared to the unmodified oligonucleotide (84). When used to treat cells other than L929 cells, however, poly-L-lysine conjugates were inactive (64).

A number of lipid conjugates have also been studied. 5' linked triethylammonium 1,2 di-O-hexadicyl-rac-glycerol-3-H-phosphonate oligonucleotides were taken up 8–10-fold more than unmodified oligonucleotides by L929 cells and were more active against varicella zoster viral infections, albeit at high concentrations (85). An oligonucleotide linked at the 5' terminus to an undecyl residue was reported to be active, but no uptake or stability studies were reported (86).

The intracellular fate of oligonucleotides injected into oocytes and the uptake of oligonucleotides into oocytes have also been studied. When injected into *Xenopus* oocytes, unmodified oligonucleotides were degraded within 1 min primarily by 3' exonuclease digestion, but other nucleolytic activities were also present (87, 88). Interestingly, in this system, even phosphorothioate oligonucleotides were reported to be degraded, albeit much more slowly than phosphodiesters (89). These observations were extended in studies on oligodeoxynucleotides injected into CV-1 endothelial cells. A 28-mer oligonucleotide of either phosphodiester, phosphorothioate, or methylphosphonate type was injected into the cytoplasm of these cells. All three types of oligonucleotides localized to the nucleus in a temperature- but not energy-dependent fashion. The methylphosphonate oligonucleotide concentrated in regions of genomic DNA, in contrast to the two other oligonucleotides that co-localized with small nuclear ribonucleoproteins (90). Uptake of unmodified oligonucleotides by pre-implantation embryos was reported to be virtually nil (91).

Liposomes and related formulations have been shown to enhance cellular uptake of oligonucleotides in vitro. Loke et al (92) compared the uptake of phosphodiester and phosphorothioate deoxythymydine heptamers into HL-60 cells by using oligonucleotides coupled to 2-methyloxy-6-chloro 9-(5-hydroxypentyl) amino acridine and monitoring with flow cytometry. They did not determine the integrity of the oligonucleotides, but reached the conclusion that phosphodiester dT_7 was taken up by HL-60 cells much more effectively than phosphorothioate d-T_7, and that uptake plateaued at 50 hr. They reported increased anti-c-*myc* activity of phosphorothioate oligonucleotides after loading them in phosphatidyl serine liposomes. The uptake of a tetramer 2'-5' deoxyadenylate into L1210 cells was reported to be increased by loading the oligo-adenylate into *Staphylococcus aureus* protein A-crosslinked phospholipid vesicles (93). In our laboratories, we have shown that lipofectin, a cationic lipid mixture, can significantly increase the uptake and activity of phosphorothioate oligonucleotides in several cell lines. It also alters the intracellular distribution of these nucleotides (78).

With the exception of methylphosphonates, the conclusion from studies that have addressed the mechanisms of uptake of oligonucleotides is that the most likely mechanism is receptor-mediated endocytosis. In fact, in one study an 80-kd protein that appeared to bind oligonucleotides was partially purified

and postulated to be a "receptor" (79). The evidence supporting this mechanism is limited, however, and data are insufficient to conclude that receptor-mediated endocytosis is the most common or only mechanism of uptake of charged oligonucleotides in most cells.

In conclusion, although many questions remain to be answered, it appears that many cells in tissue culture may take up oligonucleotides at pharmacologically relevant concentrations. Clearly, oligonucleotides of different types behave differently and there are substantial variations as a function of cell type. Moreover, length and specific sequences may alter uptake, and pendant modifications may profoundly influence cellular uptake.

Once in the cell, it would seem that oligonucleotides distribute to the cytoplasm and the nuclei. In most if not all cells, phosphodiester oligonucleotides are rapidly degraded whereas methylphosphonates and phosphorothioates are much more stable. Again, pendant modifications may alter the rate of intracellular degradation and distribution.

Mechanisms of uptake and distribution are poorly understood. Clearly, however, multiple mechanisms may play a role, and different types of oligonucleotides may behave very differently.

Novel formulations may enhance cellular uptake. Liposomes and cationic lipids significantly enhance uptake and may alter the mechanisms of uptake and intracellular fate of oligonucleotides.

IN VIVO PHARMACOKINETICS Preliminary in vivo pharmacokinetic data are now available on methylphosphonate and phosphorothioate oligonucleotides. A 12-mer ^{3}H-labeled methylphosphonate injected in the tail vein of mice was rapidly cleared as intact oligonucleotide and distributed broadly to all tissues except the brain (94).

More extensive studies have been performed on ^{35}S-labeled phosphorothioates in rats. A true distribution phase of 15–25 min was observed after a single IV dose of a 27-mer followed by a prolonged elimination phase of 20–40 hr (94). The prolonged elimination phase may result from the binding of phosphorothioates to serum proteins. Phosphorothioates distrubuted broadly to all tissues except the brain and were eliminated in the urine intact. Phosphorothioates were rapidly and extensively absorbed after IM and IP administration (94).

Repeated daily doses of 50 mg/kg of a 27-mer phosphorothioate to mice resulted in similar distribution and elimination kinetics but slight differences in tissue concentrations from single dose studies. Liver, kidney, spleen, and lung were the organs with highest concentrations. Again, the drug was excreted intact in the urine (94).

Continuous osmotic pump administration of the same compound subcutaneously for 4 wk at doses of 50–150 mg resulted in similar pharmacokinetics (94).

Studies with ISIS 1082, a 21-mer phosphorothioate, in mice showed that when applied to the cornea in a sodium acetate buffer, significant adsorption to the cornea and absorption into the aqueous and vitreous humors occurred. Moreover, significant systemic bioavailability was observed (78). In rabbit, as much as 25% of an applied ocular dose was systemically bioavailable (unpublished observations). Post absorption pharmacokinetics were equivalent to IV pharmacokinetics.

Recently, a 20-mer phosphodiester was administered intravenously to rabbits. Clearance from blood was rapid and, after 90 min, 16% of the dose was found in the urine and was intact. In blood, at least 17% of the drug was estimated to be completely degraded within 5 min (95).

Toxicology

IN VITRO

Phosphodiesters Very little information has been published on the in vitro toxicities of unmodified oligonucleotides. In most systems, the oligonucleotides are thought to be rapidly degraded. When a 15-mer complementary to a c-myc sequence was incubated with human lymphocytes at 30 μM for 4 hr, no toxicity was observed. Longer incubation (24 hr) in 10% serum resulted in reduced ^{3}H-thymidine incorporation, but the authors concluded that this was probably due to dilution of the thymidine pool by thymidine liberated after rapid degradation of the oligonucleotide (96).

The incubation of a transformed leukemic cell line with 50 μM of a 20-mer complementary to a sequence in the BCL-1 proto-oncogene was reported to result in no decrease in viability as judged by trypan blue exclusion (97).

Methylphosphonates Incubation of Vero cells with 30 μM and lower concentrations of an 8-mer methylphosphonate for 24 hr resulted in no decrease in growth rate or cell count; however, 48 hr incubation resulted in 40% inhibition of growth rate (98). Similarly, neither of three 9-mers had any effect on L929 cell plating efficiency or protein synthesis after 16 or 40 hr incubations with 150 μM of drug (99). Incubation of T15 cells with 80 μM of a 9-mer directed against N-ras for 48 hr produced no effect on protein synthesis or viability (100). Similar results were reported for HT29 cells.

Inasmuch as methylphosphonate oligonucleotides have, when they have displayed activity, effective concentrations of 50–100 μM, the therapeutic index in vitro may be rather modest. Much more detailed studies are required before reaching final conclusions, however.

Phosphorothioates Phosphorothioate oligonucleotides bind to a variety of proteins, including serum albumin. In cell free protein translation experiments, they have been shown to induce nonspecific inhibition of protein synthesis (11, 101, 102). In wheat germ and rabbit reticulocyte lysate assays,

concentrations as high as 100 nM of a 17-mer phosphorothioate targeted to the protein mRNA inhibited globin synthesis relatively specifically. At 10 μM, nonspecific effects were observed (103). The nonspecific effects of phosphorothioates in these assays are length-dependent, as a 5-mer was much less potent than the 14-mers and dC28 appeared to be the most potent phosphorothioate oligonucleotide tested. In studies in our laboratories, we have made similar observations with a number of phosphorothioate oligonucleotides (G. D. Hoke et al, unpublished observations).

Phosphorothioate oligonucleotides have also been shown to inhibit DNA polymerases, reverse transcriptases, and nucleases when incubated in cell free systems (76, 77, 104).

Despite the potential nonspecific interactions of phosphorothioate oligonucleotides with cellular proteins, a wide variety of compounds have been shown to have excellent therapeutic indices. Microinjection of nanomolar concentrations of a 17-mer into *Xenopus* oocytes inhibited β-globin synthesis. When 16 μM of the compound were injected, however, protein synthesis was aborted and the oocytes underwent extensive cytolysis (89).

Incubation of cells in vitro with phosphorothioate oligonucleotides has likewise resulted in toxicities only at concentrations much higher than those at which therapeutic activities were observed. Human mononuclear cells were unaffected after 20 hr of incubation with 25 μM of several 15-mers (105). T697 cells were unaffected by a three-day exposure to 25 μM of a 20-mer (97, 106).

In our laboratories, we have determined the effects of ISIS 1082, a 21-mer phosphorothioate that inhibits herpes simplex virus types 1 and 2 infections in HeLa cells at 200–400 nM, on HeLa cell viability, DNA synthesis, RNA synthesis, protein synthesis, and energy metabolism. At no concentration below 500 μM were statistically significant effects observed after incubation for 96 hr. Exposure of HeLa cells to 500 μM ISIS for 48 hr resulted in 20% inhibition of protein synthesis (77). Similar results were observed in other cell lines.

Table 4 presents results from studies on 20 phosphodiester or phosphorothioate oligonucleotides targeted to various regions in the 5-lipoxygenase gene. Again, most of the phosphorothioates displayed toxicities only at 50 μM and greater. The exceptions to this rule were three 30-mers that inhibited cell growth at 10–35 μM. Clearly, one can conclude from this study that toxicity was time- and concentration-dependent and that, with longer exposures in particular, phosphorothioates were more toxic than their phosphodiester analogs (73).

We have identified other factors that influence the toxicity of phosphorothioates. Cell type may alter toxicity significantly. A comparison of the toxic effects of a 15-mer phosphorothioate on HL60 cells, U937 cells, and RBL-1

Table 4 In vitro toxicities of 5-lipoxygenase oligonucleotides in HL-60 cells[a]

Compound	Oligonucleotide Class[b]	Length	Sequence	AT:GC	24 hr	48 hr	72 hr	96 hr
1787	PD	15	5'-GTGTGCCACCAGCAG-3'	1:2	21.5	18.5	16.0	14.4
1788	PS	15			>100	35.0	25.0	19.0
1789	PD	30	AATGGTGAATCTCAC		>100	>100	>100	>100
1790	PS	30	GTGTGCCACCAGCAG	1:1.1	>100	>100	15.0	11.8
1795	PD	15	TGCCAGTGATTCATG	1:0.88	63.0	39.5	34.0	26.0
1796	PS	15			>100	>100	50.0	35.0
1797	PD	30	GGTCTTCCTGCCAGT		>100	>100	50.0	74.0
1789	PS	30	GATKATGACCCGCT	1:1.31	>100	20.0	10.0	10.0
1799	PD	15	GTCCTGATGGCTTCC	1:1.5	28.0	25.0	22.0	22.0
1800	PS	15			>50.0	50.0	50.0	34.0
1801	PD	30	GTCCTGATGGCTTCC		>50.0	>50.0	>50.0	>50.0
1802	PS	30	CACACCAGGAGCCCG	1:2.0	35.0	27.0	21.0	3.9
1812	PD	15	GTTGCTCCTTGGTGT	1:1.14	29.0	17.0	16.0	18.0
1813	PS	15			>50.0	32.0	25.0	43.0
1814	PD	30	ATTTGCTGTTGCTGC		>50.0	32.0	25.0	
1815	PS	30	TTGGTGTGGAAATGC	1:0.88	10.0	9.0	10.0	43.8
1816	PD	15	AGGTGTCCGCATCTA	1:1.14	150.0	250.0	>50.0	>50.0
1817	PS	15			32.0	>50.0	>50.0	>50.0
1818	PD	30	TCGGCGCGGGGTCC	1:2.33	>50.0	>50.0	>50.0	>50.0
1819	PS	30	AGGTGTCCGCATCTA		15.0	19.0	19.0	20.0

[a] HL 60 cells were incubated in 96 well plates with increasing concentrations of oligonucleotides (1-50 or 100 μ M) in the presence of 10% fetal bovine serum. Viability of the cells was determined at each time point by crystal blue exclusion. IC_{50} values were obtained by plotting percentage control cell number vs. drug concentration.

[b] PD = Phosphodiester; PS = Phosphorothioate.

cells showed considerable variation in sensitivity; HL60 cells were the most sensitive. As phosphorothioates bind to serum albumin, in the presence of 10% fetal calf serum, a 15-mer produced no cytotoxicity after 24 hr of incubation at 100 μM. In the presence of 2.5% fetal calf serum, the IC_{50} was 19 μM. Finally, the purity of the oligonucleotide has a significant effect. Purification of oligonucleotides in triethyl ammonium buffers with trityl-on HPLC followed by removal of the trityl groups in triethyl ammonium may result in substantial contamination with triethyl ammonium ions, which are toxic to cells (73). Others have alluded to batch-to-batch variations and the potential that contaminants might contribute to toxicities, but they have not identified potential toxins (60, 97, 106–108).

Pendant group modified oligonucleotides Limited information is available concerning the effects of pendant groups on the toxicities of oligonucleotides. An acridine conjugated 7-mer phosphodiester was reported to produce no toxicities at 100 μM even though the free acridine had an IC_{50} for cell viability of 2 μM (109). Two 11-mer phosphorodiesters that were covalently attached to an undecyl group at the 5' terminus had no apparent toxic effect on MDCK cells at 100 μM (86). 5'terminal phospholipid conjugates of both phosphodiester and phosphorothioate oligonucleotides produced little toxicity in L292 cells when incubated at 50 to 100 μM (85). In contrast, a phosphodiester 15 mer linked to poly-L-lysine was toxic to L929 cells at 1 μM (84).

Table 5 summarizes published data concerning the in vitro toxicology.

IN VIVO Although only preliminary toxicologic data are available, considerably more information should soon be available, as several compounds are currently in preclinical development.

Single-dose toxicity studies in mice were reported for phosphodiester (19), methylphosphonate (110), phosphomorpholidate, and phosphorothioate oligonucleotides. Unmodified oligonucleotides resulted in deaths in two of four treated mice at 160 mg/kg and all four mice treated with 640 mg/kg IV. Within three days after injection, a phosphorothioate oligonucleotide resulted in equivalent toxicities to the phosphodiester. The other analogs produced similar toxicologic effects with slight differences in doses.

Single doses of as much as 3.5 mg of a 27-mer complementary to the REV gene of HIV given IV or IP produce no toxicities in rats. Daily injections of 50 mg/kg IV of the same compound for 12 days in mice resulted in no observable toxicities. This 27-mer was also administered via a subcutaneous osmotic pump designed to administer up to 150 mg at a constant rate for 4 wk to rats. Again, no toxicities in any organ were observed (94).

ISIS 1082, a 21-mer phosphorothioate targeted to inhibit herpes virus types 1 and 2, has been administered topically to mouse and rabbit eyes for as much as 21 days and resulted in no ocular toxicities. In rabbits, other organs were examined, and no effects were observed. Given the extensive bioavailability of ISIS 1082 in rabbits after ocular administration, this constitutes a significant observation.

Single doses of ISIS 2105, a 20-mer phosphorothioate active against human papilloma viruses, were administered intradermally and resulted in no local or systemic toxicities.

Consequently, a growing body of data supports the contention that at least single doses of phosphorothioate oligonucleotides may be given to mice, rats, and rabbits without significant acute or subacute toxicities.

MUTAGENICITY Virtually no data have been published on the potential mutagenicity of oligonucleotides. A 27-mer phosphorothioate was reported to be negative in an Ames assay in the presence or absence of a liver metabolic activation system at doses as high as 5 mg/plate (101).

P. Iverson (personal communication; 101) compared a number of oligonucleotide types and related chemicals in hamster lung fibroblasts. Unfortunately, although this study has been cited, the primary data have never been published, and thus it is difficult to draw any conclusion.

Mechanisms of Action of Oligonucleotides Interacting with Nucleic Acid Targets

The mechanisms by which interactions of oligonucleotides with nucleic acids may induce biological effects are complex and potentially numerous. Furthermore, very little is currently understood about the roles of various mechanisms or the factors that may determine which mechanisms are involved after oligonucleotides bind to their receptor sequences. Consequently, a discussion of mechanisms remains largely theoretical. Although a number of potential schemes to classify mechanisms of action might be employed, I prefer a scheme based on drug-receptor concepts.

OCCUPANCY-ONLY MEDIATED MECHANISMS Classic competitive antagonists are thought to alter biological activities because they bind to receptors, thereby preventing natural agonists from binding and inducing normal biological processes. Binding of oligonucleotides to specific sequences may inhibit the interaction of the RNA or DNA with proteins, other nucleic acids, or other factors required for essential steps in the intermediary metabolism of the RNA or its utilization by the cell.

Transcriptional arrest Oligonucleotides may bind to DNA and prevent either initiation or elongation of transcription by preventing effective binding of factors required for transcription, thus producing transcriptional arrest.

Table 5 In Vitro toxicology of antisense oligonucleotides

Oligonucleotide class	Length	Concentration	Target	Cell type	Time	Toxicity assesament	References
Phosphodiester	15	30 μM	c-*myc*	Human T cells	4 hours	Nontoxic	99
Phosphodiester	20	150 μM	βCL2	697 cells	3 days	Nontoxic	100
Phosphorothioate	20	25 μM	βCL2	697 cells	3 days	Nontoxic	
Phosphodiester	23	1-30 ng	Vg 1	Xenopus oocytes	2 days	Nontoxic @ low concentrations, i.e. <5 ng	92
Phosphorothioate	23	1–30 ng	Vg1	Xenopus oocytes	2 days	Toxic @ 15-30 ng	
Phosphodiester-acridine conjugate	7	50-100 μM	Type A influenza	MDCK	3 days	Nontoxic	112
Acridine alone		2 μM		MDCK	3 days	Toxic	
Methylphosphonate	9	150 μM	v s v	L929	16,40 hours	Nontoxic	102
Methylphosphonate	9	80 μM	N-*ras*	T15	48 hours	Nontoxic	103
Methylphosphonate	9	80 μM	N-*ras*	HT29	48 hours	Nontoxic	
Phosphodiester	14	1-25 μM	HIV	ATH8	1 days	Minor toxicity @ all conc. (<35%)	20
Methylphosphonate	14	1-25 μM			7 days	Minor toxicity @ all conc. (<27%)	
Phosphorothioate (heteropolymer)	14	1-25 μM			7 days	Nontoxic	
Phosphorothioate (homopolymer)	14-28	1-25 μM			7 days	Nontoxic	76
Phosphorothioate (homooligomer)	28	3-50μM	HSV-2	HeLa S_3	72 hours	Nontoxic	
Phosphorothioate (antisense)	20	4-100 μg/ml	HIV	H9, MOLT3	96 hours	Nontoxic	19
Phosphorothioate (nonsense)	20	4-100 μg/ml		H9, MOLT3	96 hours	Nontoxic (4 μg/ml) Toxic (20 μg-5% 100 μg-67%)	
Phosphodiester homooligomer (dT)	15	4-100 μg/ml		H9, MOLT3	96 hours	Toxic	
Phosphodiester homooligomer (dA)	15	4-100 μg/ml		H9, MOLT3	96 hours	Minor toxicity	
Phosphormorpholidate homooligomer (dT)	15	4-100 μg/ml		H9, MOLT3	96 hours	Toxic	
Phosphormorpholidate homooligomer (dG)	15	4-100 μg/ml		H9, MOLT3	96 hours	Toxic	
Phosphorothioate	17	16 μM	β-Globin	*Xenopus* oocytes	>6 hours	Toxic	106
Phosphorothioate	15	0.1–25 μM	ILIβ	Human blood monocytes	20 hours	Nontoxic	108

It is possible that oligonucleotides could bind to segments of DNA that are partially denatured by the transcription complex, although this is highly unlikely. The initiation and elongation of transcription require a complex set of proteins and other factors, and it is difficult to conceive of a mechanism by which oligonucleotides might compete effectively against the transcriptional machinery for these single-stranded regions. Nevertheless, despite the improbability of such an event, reports of activities have been made that can be explained most simply by this mechanism (112, 113). Additionally, Helene and colleagues (114) reported that hexanucleotides to nonanucleotides with acridine derivatives at the 3' terminus inhibited transcription of the β-lactamase gene. When the RNA polymerase was preincubated with the oligonucleotide-acridine adducts, however, they observed nonspecific inhibition (115).

The alternative to seeking transient single-stranded regions or to attempting to denature a double-stranded region of DNA is to inhibit transcription by interacting with double-stranded DNA, i.e. forming triple-stranded structures. To form triple-stranded structures, hydrogen bonds other than Watson-Crick must be formed. In most current triple–strand motifs, the oligonucleotide becomes the third strand by recognizing hydrogen bonding donor/receptor sites on a purine reference strand and lying in the major groove (116–124). Alternative motifs have also been proposed. For example, Hogan and colleagues (125) proposed that a purine-rich oligonucleotide can form a triplex structure based upon the purines in the oligonucleotide base pairing in parallel fashion with the purines in the duplex DNA. Studies by Dervan's group (126), however, suggested that the purine-rich oligonucleotide bound to the duplex DNA with an antiparallel orientation.

The formation of triple-stranded structures by using natural nucleosides requires runs of purines Watson-Crick-hydrogen-bonded to their complementary pyrimidines. When cytidine is used to form a triple strand with a G-C base pair, it must be protonated; this occurs at nonphysiological acidic conditions (121). Furthermore, all motifs employ one or more "weak" hydrogen bonds. Thus, to achieve sufficient stability, relatively long triple-strand structures are required.

The principal theoretical advantage of triple helical inhibition schemes is that transcription represents the first step in the intermediary metabolism of RNA and may, therefore, provide substantial leverage for drug therapy. The other advantages that have been suggested are much more speculative. For example, it has been suggested that the smaller number of genes (one or two) compared to the number of mRNA molecules (usually less than 1000) per cell is an advantage for approaches that inhibit transcription. This suggestion ignores the kinetics of the targets, however. Genes have an infinite half-life relative to cell life. RNA molecules are synthesized and degraded with

varying kinetics. Furthermore, a variety of mechanisms exist to assure that even covalent modifications of DNA are repaired. Another concept has been that triple helixes in DNA might produce permanent biological effects. That even alkylating and DNA-cleaving anticancer drugs do not produce permanent effects points to the speciousness of this notion.

A number of theoretical disadvantages of triple helical inhibition of transcription have also been enumerated. Sequence specific binding is not yet possible, as runs of homopyrimidines are required. These sequences may play important regulatory roles in DNA, as they are much more abundant than statistically predicted (16). Longer term, a more substantial problem may simply be gaining sequence-specific access to DNA in chromatin. Additionally, deliberate interactions with the genome raise concerns about mutagenicity, carcinogenicity, and teratogenicity, which, in most therapeutic settings, are of considerable importance.

Several strategies have been developed to circumvent the requirement for purine-pyrimidine runs and other limitations. For example, purine oligonucleotides form triplex structures at higher pH values than pyrimidine-rich oligonucleotides (125, 126). Similarly, pyrimidine-rich oligonucleotides, in which 2'-O-methyl pseudoisocytidine was substituted for 2' deoxycytidine, formed triplex structures as neutral pH (127). Oligonucleotides with linkers that allow crossover of the oligopyrimidine from one strand of the duplex to the other have been reported and this motif suggested to be a solution to a broader sequence repertoire (128). To enhance the stability of triple helices, intercalators and photoactivatable crosslinkers and alkylators have been conjugated to oligo pyrimidines (129–131). To increase potency and enable identification of sites of binding, a number of cleavage moieties have been conjugated to oligopyrimidines (132–137). Finally, to enhance nuclease stability, methylphosphonates (138) and α-oligonucleotides (136) have been shown or suggested to form triple helices.

In addition to cleavage of DNA in vitro by triplex-forming oligonucleotides coupled to cleavage reagents and alkylation induced by oligonucleotide-coupled alkylators, several other methods have been used to show triplex formation. These include agarose affinity column purification (139), NMR (140), protection from uv dimerization (141), solution hybridization (142), inhibition of binding of DNA-binding proteins (143), inhibition of restriction endonucleases (144), and repression of c-myc transcription in vitro (125). Recently, a 28-mer phosphodiester stabilized at the 3' end by alanine and directed to enhancer elements for the IL-2 receptor gene was shown to inhibit the transcription of the gene when incubated with human lymphocytes. The authors reported evidence for selectivity to oligonucleotides as well (145).

Obviously, triple-helix-based inhibition of transcription is of potential therapeutic importance, particularly for targets that for a variety of reasons

may be difficult to inhibit at the post-transcriptional level. Substantial medicinal chemistry must be completed, however, to create oligonucleotides that can interact with duplex structures in a sequence-specific fashion without requiring special motifs. Once this is accomplished, of course, additional studies must show that the other theoretical limitations discussed above can be overcome.

Inhibition of splicing A key step in the intermediary metabolism of most mRNA molecules is the excision of introns. These "splicing" reactions are sequence-specific and require the concerted action of spliceosmes. Consequently, oligonucleotides that bind to sequences required for splicing may prevent binding of necessary factors or physically prevent the required cleavage reactions. This then would result in inhibition of the production of the mature mRNA. Although there are several examples of oligonucleotides directed to splice junctions, none of the studies presented data showing inhibition of RNA processing, accumulation of splicing intermediates, or a reduction in mature mRNA. Nor are there published data in which the structure of the RNA at the splice junction was probed and the oligonucleotides demonstrated to hybridize to the sequences for which they were designed (146–149). Activities have been reported for anti-c-*myc* and antiviral oligonucleotides with phosphodiesters, methylphosphonates, and phosphorothioates.

Translational arrest Without question, the mechanism for which the majority of oligonucleotides have been designed is translational arrest. Oligonucleotides have been designed to bind to the translational initiation codon. The positioning of the initiation codon within the area of complementarity of the oligonucleotide and the length of the oligonucleotide used have varied considerably. Again, unfortunately, only in relatively few studies have the oligonucleotides been shown to bind to the sites for which they were designed, and other data that support translation arrest as the mechanism reported.

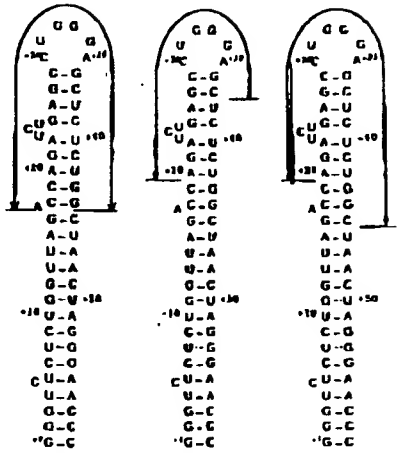
Target RNA species that have been reported to be inhibited include HIV (19), vesicular stomatitus virus (VSV) (82), n-*myc* (150), and a number of normal cellular genes (151–154).

In our laboratories, we have shown that a significant number of targets may be inhibited by binding to translation initiation codons. For example, ISIS 1082 hybridizes to the AUG codon for the UL13 gene of herpes virus types 1 and 2. Studies with RNaseH confirmed that ISIS 1082 binds selectively in this area. In vitro protein synthesis studies confirmed that ISIS 1082 inhibited the synthesis of the UL13 protein, and studies in HeLa cells showed that it inhibited the growth of herpes type 1 and type 2 with an IC_{50} of 200–400 nm

by translation arrest (155). Similarly, ISIS 1753, a 30-mer phosphorothioate complementary to the translation initiation codon and surrounding sequences of the E2 gene of bovine papilloma virus, was highly effective, and its activity was shown to be due to translation arrest. ISIS 2105, a 20-mer phosphorothioate complementary to the same region in human papilloma virus, was shown to be a very potent inhibitor. Compounds complementary to the translation initiation codon were the most potent of the more than 50 compounds studied complementary to various other regions in the RNA (156).

In conclusion, translation arrest represents an important mechanism of action for antisense drugs. A number of examples purporting to employ this mechanism have been reported. Recent studies on several compounds have provided data that unambiguously demonstrate that this mechanism can result in potent antisense drugs.

Disruption of necessary RNA structure RNA adopts a variety of three-dimensional structures induced by intramolecular hybridization, the most common of which is the stem loop (Figure 2). These structures play crucial roles in a variety of functions. They are used to provide additional stability for



length	28	18	26
P=S	1972	1308	1307
P=O	1971	1116	1118

Figure 2 Antisense oligonucleotides directed against the HIV TAR element. The oligonucleotide sequences are complementary to the tar sequences where indicated.

RNA and as recognition motifs for a number of proteins, nucleic acids, and ribonucleoproteins that participate in the intermediary metabolism and activities of RNA species. Thus, given the potential general activity of the mechanism, it is surprising that occupancy-based disruption RNA has not been more extensively exploited.

As an example, we designed a series of oligonucleotides that bind to the important stem-loop in all RNA species in HIV, and TAR element. We synthesized a number of oligonucleotides designed to disrupt TAR, and showed that several indeed did bind to TAR, disrupt the structure, and inhibit TAR-mediated production of a reporter gene (157). Futhermore, general rules useful in disrupting stem-loop structures were developed as well.

Although designed to induce relatively nonspecific cytotoxic effects, two other examples are noteworthy. Oligonucleotides designed to bind to a 17 nucleotide loop in *Xenopus* 28S RNA, required for ribosome stability and protein synthesis, inhibited protein synthesis when injected into *Xenopus* oocytes (158). Similarly, oligonucleotides designed to bind to highly conserved sequences in 5.8S RNA inhibited protein synthesis in rabbit reticulocyte and wheat germ systems (159).

OCCUPANCY-ACTIVATED DESTABILIZATION RNA molecules regulate their own metabolism. A number of structural features of RNA are known to influence stability, various processing events, subcellular distribution, and transport. As RNA intermediary metabolism is better understood, many other regulatory features and mechanisms will probably be identified.

5' Capping A key early step in RNA processing is 5' capping (Figure 1). This stabilizes pre-mRNA and is important for the stability of mature mRNA. It also is important in binding to the nuclear matrix and nuclear transport of mRNA. As the structure of the cap is unique and understood, it presents an interesting target.

Several oligonucleotides that bind near the cap site have been shown to be active, presumbly by inhibiting the binding of proteins required to cap the RNA. Again, however, this putative mechanism has not been rigorously demonstrated in any published study. In fact, none of the oligonucleotides have been shown in any published study to bind to the sequences for which they were designed. For example, the synthesis of SV40 T-antigen was reported to be most sensitive to an oligonucleotide linked to polylysine and targeted to the 5' cap site of RNA (160).

In studies in our laboratory, we have designed oligonucleotides to bind to 5' cap structures and reagents to specifically cleave the unique 5' cap structure (161).

Inhibition of 3' polyadenylation In the 3' untranslated region of pre-mRNA molecules, there are sequences that result in the post-transcriptional addition of long (hundreds of nucleotides) tracts of polyadenylate. Polyadenylation stabilizes mRNA and may play other roles in the intermediary metabolism of RNA species. Theoretically, interactions in the 3' terminal region of pre-mRNA could inhibit polyadenylation and destabilize the RNA species. Although there are a number of oligonucleotides that interact in the 3' untranslated region and display antisense activities, to date no study has reported evidence for alterations in polyadenylation.

Other mechanisms In addition to 5' capping and 3' adenylation, clearly other sequences in the 5' and 3' untranslated regions of mRNA affect the stability of the molecules. Again, a number of antisense drugs may work by these mechanisms.

Zamecnik & Stephenson (1) reported that a 13-mer targeted to untranslated 3' and 5' terminal sequences in Rous sarcoma viruses was active. Oligonucleotides that were conjugated to an acridine derivative and targeted to a 3'-terminal sequence in type A influenza viruses were reported to be active (109, 162, 163). Against several RNA targets, studies in our laboratories have shown that sequences in the 3' untranslated region of RNA molecules are often the most sensitive. For example, ISIS 1939, a 20-mer phosphorothioate that binds to and appears to disrupt a predicted stem-loop structure in the 3' untranslated region of the mRNA for ICAM, is a potent antisense inhibitor. However, inasmuch as a 2'-O-methyl analog of ISIS 1939 was much less active, it is likely that in addition to destabilization to cellular nucleolytic activity, activation of RNase H (see below) is also involved in the activity of ISIS 1939 (164).

ACTIVATION OF RNase H RNase H is an ubiquitous enzyme that degrades the RNA strand of an RNA-DNA duplex. It has been identified in organisms as diverse as viruses and human cells (for review see 165). At least two classes of RNase H have been identified in eukaryotic cells. Those in yeast and multiple enzymes with RNase H activity have been observed in prokaryotes (165). Furthermore, data suggest that there are multiple isozymes in eukaryotic cells.

Although RNase H is involved in DNA replication, it may play other roles in the cell and is found in the cytoplasm as well as the nucleus (166). The concentration of the enzyme in the nucleus is thought to be greater, however, and some of the enzyme found in cytoplasmic preparations may be due to nuclear leakage.

RNase H activity is quite variable. It is absent or minimal in rabbit reticulocytes (167) but present in wheat germ extracts (165) in a wide range of

cells (16). The level of RNase H varies as a function of development, differentiation, and rate of cell division (165). In HL60 cells, for example, the level of activity in undifferentiated cells is greatest; it is relatively high in DMSO and vitamin D–differentiated cells, and much lower in PMA-differentiated cells (G. D. Hoke et al, unpublished observations).

The precise recognition elements for RNase H are unknown; however, it has been shown that oligonucleotides with DNA-like properties as short as tetramers can activate RNase H (168). Changes in the sugar influence RNase H activation, as sugar modifications that result in RNA-like oligonucleotides, e.g. 2'-fluoro or 2'-O-methyl, do not appear to serve as a substrate for RNase H (44, 169). Alterations in the orientation of the sugar to the base can also affect RNase H activation, as α-oligonucleotides are unable to induce RNase H or may require parallel annealing (41, 170). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates are not substrates for RNase H (74, 143). In contrast, phosphorothioates are excellent substrates (106, 155, 171; G. D. Hoke unpublished observations). More recently, chimeric molecules have been studied as substrates for RNase H (172, 173). A single ribonucleotide in a sequence of deoxyribonucleotides was recently shown to be sufficient to serve as a substrate for RNase H when bound to its complementary deoxyoligonucleotide (174).

Despite the information about RNase H and the demonstrations that many oligonucleotides may activate RNase H in lysate and purified assays (168, 175–177), relatively little is known about the role of structural features in RNA targets in activating RNase H. There is little direct proof that RNase H activation is, in fact, the mechanism of action of oligonucleotides in cells. Recent studies in our laboratories provide additional, albeit indirect, insights into these questions. ISIS 1939 is a 20-mer phosphorothioate complementary to a sequence in the 3' untranslated region of ICAM-1 RNA. It inhibits ICAM production in human umbilical vein endothelial cells, and northern blots demonstrate that ICAM-1 mRNA is rapidly degraded. A 2'-O-methyl analog of ISIS 1939 displays higher affinity for the RNA than the phosphorothioate, is stable in cells, but inhibits ICAM-1 protein production much less potently than ISIS 1939. It is likely that ISIS 1939 destabilizes the RNA and activates RNase H. In contrast, ISIS 1570, an 18-mer phosphorothioate that is complementary to the translation initiation codon of the ICAM-1 message, inhibited production of the protein but caused no degradation of the RNA. Thus, two oligonucleotides that are capable of activating RNase H had different effects, depending on the site in the mRNA to which they bound (164).

COVALENT MODIFICATION OF THE TARGET NUCLEIC ACID BY THE OLIGONUCLEOTIDE A large number of oligonucleotides conjugated to alkylating and photoactive alkylating species have been synthesized and tested for effects on purified nucleic acids and intracellular nucleic acid targets (162, 178). The potential advantage of such modifications is, of course, enhanced potency. The potential disadvantages are equally obvious: nonspecific alkylation in vivo and resulting toxicities.

A variety of alkylating agents have been used to modify single-stranded DNA covalently and have been shown to induce alkylation at sequences predicted by the complementary oligonucleotide to which they were attached (178–182). Similar alkylators have been employed to modify double-stranded DNA covalently after triplex formation (125, 137, 183, 184).

Photoactivatable crosslinkers and platinates have been coupled to oligonucleotides and shown to crosslink sequence-specifically as well. Photoactivatable crosslinkers coupled to phosphodiesters, methyl-phosphonates, and phosphorothioates have been shown to produce sequence-specific crosslinking (59, 130, 185–190). Photoreactive crosslinking has also been demonstrated for double-stranded DNA after triplex formation (136, 191).

Preliminary data suggesting that covalent modifications of nucleic acids in cells is feasible and may enhance the potency of oligonucleotides have also been reported. Psoralen-linked methylphosphonate oligonucleotides were reported to be significantly more potent than methylphosphonate oligonucleotides in inhibiting rabbit globin mRNA in rabbit reticulocyte lysate assay (33). Psoralen-linked methylphosphonates were also reported to be more potent in inhibiting herpes simplex virus infection in HeLa cells in tissue culture (147). Additionally, although it did not produce covalent modification, a 9-mer phosphodiester conjugated with an intercalator inhibited mutant Ha-ras synthesis in T-24 bladder carcinoma cells (81).

OLIGONUCLEOTIDE-INDUCED CLEAVAGE OF NUCLEIC ACID TARGETS Another attractive mechanism by which the potency of oligonucleotides might be increased is to synthesize derivatives that cleave their nucleic acid targets directly. Several potential chemical mechanisms are being studied, and positive results have been reported.

The mechanism that has been most broadly studied is to conjugate oligonucleotides to chelators of redox-active metals and generate activated oxygen species that can cleave nucleic acids. Dervan and colleagues have developed EDTA-conjugated oligonucleotides that cleave double-stranded DNA sequence specifically after triplex formation (124, 137). Dervan and others have employed EDTA-oligonucleotide conjugates to cleave single-stranded DNA (192, 193). It is thought that EDTA chelates iron, which generates hydroxyl radicals that cleave the DNA; however, the cleavage occurs at several nucleotides near the nucleotide at which EDTA is attached.

In the presence of copper, oligonucleotides that are conjugated to 2,10-phenantholine also cleave DNA with some sequence specificity (129,

133–135, 194, 195), as do porphyrin-linked oligonucleotides when exposed to light (196–198). Phorphyrin-linked oligonucleotides, however, oxidize bases and induce crosslinks as well as cleave the phosphodiester backbone.

To date, no reports have demonstrated selective cleavage of an RNA or enhanced potency of oligonucleotides in cells using oligonucleotides and cleaving moieties that employ these mechanisms. Studies in progress in a number of laboratories will probably soon explore this question.

Another mechanism that may be intrinsically more attractive for therapeutic applications, particularly for cleavage of RNA targets, is a mechanism analogous to that used by many ribonucleases, nucleotodiyltransferases, phosphotransferases, and ribozymes.

Ribozymes are oligoribonucleotides or RNA species capable of cleaving themselves or other RNA molecules (199). Furthermore, the Tetrahymena ribozyme has been shown to cleave DNA, but at a slower rate than RNA (200). Although several classes of ribozymes have been identified that differ with regard to substrate specificity, the use of internal or external guanosine, and other characteristics, they all employ similar enzymatic mechanisms. Cleavage and ligation involve a Mg^{2+}-dependent transesterification with nucleophilic attack by the 3'-hydroxyl of guanosine (200).

The notion that we might design a relatively small ribozyme that could interact with desired sequences as a therapeutic was given impetus by studies that showed activity for ribozymes as short as a 19-mer (201) and by the demonstration that ribozyme activity can be retained after substitutions such as phosphorothioates are introduced (200).

Other approaches to creating oligonucleotides that cleave RNA targets are to synthesize oligonucleotides with appropriate adducts positioned to catalyze degradation via acid-base mechanisms. Substantial progress is being made in this area as well (P. D. Cook et al, unpublished observations).

A few studies have attempted to compare activities of oligonucleotides targeted to different receptor sequences in the same RNA. In our laboratories, we have shown that the most sensitive site in ICAM mRNA appears to be the 3' untranslated region (164). Against PLA_2, the most active molecules are also directed to sequences in the 3' untranslated region. In contrast, the most active molecules against ELAM are in the 5' untranslated region (C. F. Bennett et al, unpublished observations). However, oligonucleotides directed to the 5' cap site, translation initiation codon, and coding regions have also shown activity (for review see 202, 203).

In conclusion, an array of potential post-binding mechanisms have already been identified for oligonucleotides. For specific oligonucleotides, however, insufficient data are available to draw firm conclusions about mechanisms. More than one mechanism may very well play a role in the activity of a given oligonucleotide. Many additional mechanisms are likely to be identified as progress continues. It is important to consider the structure and function of receptor sequences in designing oligonucleotides and to continue to study potential mechanisms in detail. Clearly, RNase H may play a role in the mechanisms of many oligonucleotides, but, equally clearly, it is not critical for the activity of others. In the future, the mechanisms for which oligonucleotides are designed will probably be optimized for each target and class of oligonucleotide.

MEDICINAL CHEMISTRY

The core of any rational drug discovery program is medicinal chemistry. Although the synthesis of modified nucleic acids has been a subject of interest for some time, the intense focus on the medicinal chemistry of oligonucleotides dates perhaps to no more than three years prior to this writing. Consequently, the scope of medicinal chemistry has recently expanded enormously, but the biological data to support conclusions about synthetic strategies are only beginning to emerge. As several excellent reviews have been published recently, I focus here strictly on design features and progress in evaluating various approaches to enhance the properties of oligonucleotides as drugs (12, 16, 202, 203).

Modifications in the base, sugar, and phosphate moieties of oligonucleotides have been reported. The subject of medicinal chemical programs include approaches to create enhanced affinity and more selective affinity for RNA or duplex structures; the ability to cleave nucleic acid targets; enhanced nuclease stability, cellular uptake, and distribution; and in vivo tissue distribution, metabolism and clearance.

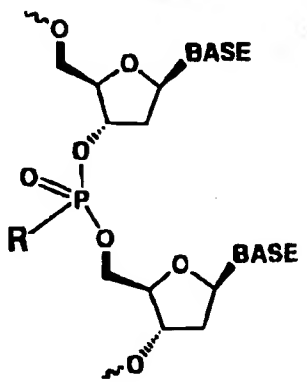
Modifications of the Phosphate

Table 6 shows the structures of various phosphate analogs. The properties of phosphodiester, phosphorothioate, and methylphosphonate analogs have been discussed extensively. More recently, phosphorodithioates have been synthesized and reported to be nuclease-resistant and to form stable duplexes with complementary DNA (204). Of course, a potential advantage of the phosphorodithioates is the lack of a chiral center. Another interesting recent modification is the replacement of one of the nonbonding oxygen atoms with a borane group (205). A dimer was reported to be nuclease-resistant, but little additional information is available.

Modifications of the Pentofuranose Linker

Modifications of oligonucleotides that replace phosphorous may be attractive because they support the design of oligonucleotides that may have a range of

Table 6 Phosphate modifications and analogs



Structure	R	Name
1	O^-	Phosphodiester
2	S^-	Phosphorothioate
3	Me	Methyl phosphonate
4	N (alkyl)	Phosphoramidate
5	S and O → S	Phosphorodithioate
6	H_3B	Boranophosphate

charges from nonionic to negatively or positively charged. In theory, reducing the anionic character of oligonucleotides may enhance hybridization and pharmacokinetic properties.

As discussed above, earlier modifications were not specifically directed to the needs of oligonucleotide drugs. Recent modifications have attempted to maintain the geometry and spacing required to support hybridization. Replacement of the phosphorous dioxygen moiety with a methylene group has been reported by two groups (51–53). This "formacetal" linkage forms a stable duplex with DNA and to be nuclease-resistant, but it is not amenable to additional modifications without creating chiral centers and would result in a water-insoluable molecule if fully substituted throughout an oligonucleotide. Other one-for-one substitutions have been reported, but the substitutions are either less interesting or have not been evaluated sufficiently to determine their potential (for review see 12).

More complex substitutions have also been reported recently, including two atom substitutions in which the phosphorous and 5' oxygen atoms were replaced by a sulfonamide linkage (54) or methyl sulfoxyl linkage (206). A thymine tetramer in which the phosphate backbone structure was replaced with dimethylene sulfonate was recently found to hybridize with natural oligonucleotides (207). Additionally, an acetamide group has been substituted for the backbone phosphate structure in a dinucleoside (207). Finally, a great many other substitutions in the backbone have been made and will probably be published in the next year, so the repertoire of compounds should continue to increase.

Pentofuranose (Sugar) Modifications

A significant number of modifications have recently been reported. In essence, these derive from two strategies with different objectives. Oligonucleotides in which the sugars are modified uniformly throughout are designed to enhance affinity to RNA targets by facilitating the formation of a more stable helix. They also may enhance nuclease stability and membrane permeability, but these outcomes are usually secondary to the hybridization goals. In contrast, pendant modifications have also been synthesized primarily to enhance pharmacokinetics or to introduce alkylating or cleaving moieties. In any event, the sugar at the 2' position is clearly an attractive site for medicinal chemistry. Some of the properties of a few of the 2' modified oligonucleotides are shown in Table 3. This remains a fertile area for medicinal chemistry, and additional advances are likely.

More substantive alterations in sugar and even replacement of sugar are also possible. Of course, α-oligonucleotides represent one type of modification (41), but numerous other approaches are feasible. Carbocyclic (49, 50) and acyclic (47, 48) structures have been reported.

Heterocyclic Modifications

Numerous heterocyclic modifications have also been described. Many of these have been designed to enhance affinity and/or alter specificity (224). Other modifications have been developed to attach pendant modifications that may alkylate, intercalate, or cleave, as well as others that may enhance pharmacokinetic properties.

Conclusions

In conclusion, it is clear that an enormous scope for medicinal chemistry exists and that the major programs are already beginning to pay dividends.

ACTIVITIES OF OLIGONUCLEOTIDES

In the past several years, scores of articles have been published demonstrating the activity of a large number of oligonucleotides in a variety of systems. A number of excellent reviews have summarized the activities of these compounds (16, 202, 203, 208). The activities of oligonucleotides in assays of cell-free protein synthesis and after injection into cells of several types have also been summarized. Consequently, I provide a brief summary of the activities of oligonucleotides in cell-based assays and a comment on the limited in vivo data reported to date.

Activities in Cells in Tissue Culture

To date, oligonucleotides have been found to inhibit the growth of a large number of viruses in tissue culture, the expression of numerous oncogenes, a variety of normal cellular genes, and a number of transfected reporter genes controlled by several regulatory elements. The oligonucleotides used, the cells employed, and the receptor sequences, concentrations, and conditions have differed widely. Only a few of the studies have reported detailed dose response curves and conditions. Studies for which sufficient information was presented are summarized in Table 7.

The data presented in Table 7 support only a few generalizations, as follows:

1. Even though phosphodiesters are relatively rapidly degraded, a number of laboratories have reported activities for unmodified phosphodiester oligonucleotides in cells incubated in the absence of serum. The concentrations required to display activity were typically greater than 10 μM.
2. A variety of modified oligonucleotides have been reported to be active. Methylphosphonates appear to be less potent than phosphorothioates, but considerable variation has been noted, depending on the system. Conjugation of alkylators and intercalators to phosphodiesters and methylphosphates increases potency. Lipophilic and poly-lysine conjugates have also displayed enhanced activities.
3. Oligonucleotides have demonstrated a broad array of activities against viral targets, oncogenes, normal host gene products, and various transfected genes. Thus, clear evidence supports the broad, potential applicability of these drugs.
4. Although the data from studies incorporated in Table 7 are limited, when combined with the in vitro toxicologic data, the therapeutic indexes of phosphorothioates appear to be quite high in vitro. Methylphosphonates appear to have lower therapeutic indexes. Too few data are available to draw conclusions about other classes of oligonucleotides.
5. Very few data support putative mechanisms of action, and generalizations concerning desired mechanisms of action are not possible. Nevertheless, a variety of mechanisms of action may be employed by oligonucleotides to result in significant biological activities.

In Vivo Activities

Two earlier investigations have suggested in vivo activities of antisense drugs against viral infections. Although no data were reported, a methylphosphonate oligonucleotide was indicated to have been active in a mouse model of herpes simplex virus I infection (147). Another study claimed in vivo activity against tick-borne encephalitis virus (213).

Table 7 Summary of antisense oligonucleotide Activities (in vitro)[1]

Target	Cell type	Serum	Oligo types	Length	Concentration	Reference
Viruses						
HTLV-III	H9 cells	–	P	12–26	5–50 mg/ml	148
HIV	H-T cells	+	PS	14–28	0.5 1 μM	20
HIV (gag/pol)	H-T cells	+	PS	18-24	1–10 μM	211
HIV	H9 cells	+	PS, others	20	4–20 μg/ml	19
HIV	CZM cells	+	PS	18–28	10 μM	157
Herpes simplex	Vero cells	+	CH_3P	7	50–100 μM	98
Herpes simplex	HeLa cells	+	PS	28	1–10 μM (non-antisense)	76
Herpes simplex	Vero cells	+	CH_3P	12	20–50 μM	15
Herpes simplex	Vero cells	+	CH_3P psoralen	12	5 μM	15
Herpes simplex	HeLa cells	+	PS	21	0.2–4 μM	155
Vesicular stomatitis	L929 cells	+	CH_3P	9	25–50 μM	99
Vesicular stomatitis	L929 cells	+	P-lipid	11	50–150 μM	85
Vesicular stomatitis	L929 cells	+	P-poly l-lysine	10–15	0.1 μM	212
Influenza	MDCK cells	+	P-acridine	11	50 μM	109
Tick-borne encephalitis	—	+	Various	Var.	0.1–1 μM	213
SV40	MDCK cells	+	CH_3P	6–9	25 μM	214
Rous	Chicken fibroblasts	+	Various	Var.	10 μM	1
Hepatitis B	Alexander	+	P	15	8.5 μM	215
Bovine papilloma virus	C-127 cells	+	PS	4–30	0.01–1 μM	209
Oncogenes						
c-*myc*	T-lymphocytes	+	P	15	30 μM	96
c-*myc*	HL-60 cells	+	P, PS	15	10 μM	216
c-*myc*	Burkitt cells	–	P	21	100 μM	146

c-myb	PMBC	+	P	18	40 mg/ml	217
BCL-2	L697 cells	−	P, PS	20	25–150 μM	97
N-*myc*	Neuroblastoma cells	+	P	15	1–5 μM	155
N-*ras*	T15 cells	+	CH_3P	9	Inactive	100
Host genes						
Multiple drug Resistance	MCF-1 cells	+	PS	15		218
PCNA (cyclin)	3T3	+	P	18	30 μM	219
Prothymosin	Human myeloma cells		P	22		152
T cell receptor	T cells	+	P	22		153
Gm CSF	Endothelial cells	−	P	15, 18	10^{-9}M	153
CSF-1	FL-*ras*/*myc* cells	+	P	?	?	220
EGF receptor		+	P	13	30 μM	221
BFGF	Human astrocytes	−	P	15	10-75 μM	229
β Globin	Rabbit reticulocytes	+	CH_3P	9	100 μM	222
TAU	Neurons	−	P	20-25	3-50 μM	228
CAMP-Protein kinase II β	HL-60 cells	+	P	21	15 μM	210
Myeloblastin	HL-60 cells	+	P	18	?	227
Phospholipase A_2 activating protein	$BC3H_1$	+	P	25	25 μM	233
ICAM-1	A549 HVEC lymphocytes	−	PS	18-20	0.01-1 μM	154
IL-2	T-lymphocytes	−	P	15	5 μM	72
IL-1α	HUVEC	+	P	18	10 μM	154
IL-1β	Monocytes	+	PS	15	0.1-2.5 μM	105

Table 7 (*Continued*)

Target	Cell type	Serum	Oligo types	Length	Concentration	Reference
IGF-1	Myoblasts	−	P	15	10 μM	225
Perforin	T-lymphocytes	−	P	18	5-35 μM	226
Other						
Chloramphenicol acetyl transferase	CV-1 cells	+	P, PS, CH_3P	21	5-30 μM	75
Placental alkaline phosphatase driven by HIV TAR	SK-mel-2 cells	+	PS	18-28	0.25-5 μM	157
Chloramphenicol Acetyl transferase Driven by human papilloma virus E2 responsive element	C-127 and CV-1 cells	+	PS	14–20	1–10 μM	209

[a] Abbreviations: cAMP, cyclic AMP; CH_3-P, methylphosphonate oligonucleotides; EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HB, hepatitis B; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-cell lymphotrophic virus; IV, influenza virus; P, phosphodiester oligonucleotides; P-acridine, phosphodiester oligonucleotide conjugated with acridine moiety; P-lipid, phosphodiester oligonucleotide conjugated with lipid moiety; P-S, phosphorothioate oligonucleotides; PCNA, proliferating cell nuclear antigen; PMA, phorbol myristic acid; RSV, Rous sarcoma virus; TAR, Tat response element; TBE, tick-borne encephalitis; VSV, vesicular stomatitis.

Topical application of ISIS 1082 in an aqueous buffer to the cornea of mice infected with herpes virus I inhibited viral growth in a concentration-dependent fashion and cured the infection at concentrations greater than 1% (230). The activity of ISIS 1082 was equivalent to trifluorothymidine and was associated with no local or systemic toxicities.

CONCLUSIONS

Oligonucleotides designed to interact with nucleic acid receptors represent a potentially revolutionary advance in pharmacotherapy. Advances in the recent past and the intense, current focus assure that the paradigm will be fully explored.

Oligonucleotides have already been shown to work in vitro and have proven to be invaluable pharmacologic tools. The progress in resolving the basic pharmacological questions relating to oligonucleotide therapeutics and in resolving issues that will influence the commercialization of new drugs of this class has been impressive. Moreover, advances in medicinal chemistry are exciting and argue that exciting new classes of these drugs are forthcoming.

Much remains to be learned; a great deal remains to be accomplished before the paradigm is fully proven and the opportunity it represents realized. In the coming years, the key tasks will be (*a*) to place oligonucleotide therapeutics on a solid pharmacological footing by performing careful dose response curves in well-designed experiments, and (*b*) to advance the development of oligonucleotide pharmaceutic products to the point that the paradigm can be tested in man.

There is now cause for considerable optimism that the promise of oligonucleotide therapeutics may be realized.

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PHARMACOLOGY OF PROTEIN KINASE INHIBITORS

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KEY WORDS: cAMP-dependent protein kinase, cGMP-dependent protein kinase, protein kinase C, Ca^{2+}/calmodulin kinase II inhibitor, protein phosphorylation

INTRODUCTION

A complete understanding of the organization and functioning of the second messenger system requires the expertise and cooperation of several different scientific disciplines, such as molecular pharmacology, genetic manipulation, biochemistry, and cell biology. The advent of a new class of effective pharmacological agents is always an event of considerable interest, in particular when this class consists of new types of antagonists that act by specifically blocking one or more of the steps in intracellular signaling systems (1). Although various aspects of protein-phosphorylation systems have been investigated, uncertainties concerning the complex cellular responses in the second messenger system remain (2–4). Improved and sophisticated methods must be designed to estimate changes in the activities of cellular response elements after extracellular stimuli. While our comprehension of the biochemistry and molecular biology of protein kinases has progressed, the function of these enzymes in intact cells has been much more difficult to understand. For this reason researchers studying second messenger systems have long sought the development of specific and effective protein kinase inhibitors that would permit the definitive determination of the physiological role of the protein kinases (5). Protein kinase inhibitors can be used to determine the physiological significance of the protein phosphorylation systems in various types of cells. To elucidate the physiological function of each

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Disposition of the ^{14}C-Labeled Phosphorothioate Oligonucleotide ISIS 2105 after Intravenous Administration to Rats

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ABSTRACT

5'-TTGCTTCCATCTTCCTCGTC-3' (ISIS 2105) is a phosphorothioate oligodeoxynucleotide currently being evaluated as an intralesional antiviral drug for the treatment of genital warts that are caused by the human papillomavirus. ISIS 2105, labeled with ^{14}C (at the carbon-2 position of thymine) was administered as a single i.v. injection (3.6 mg/kg) to female Sprague-Dawley rats to assess the disposition of the drug. After i.v. administration of [^{14}C]2105, blood radioactivity disappeared in a multiexponential manner with the half-lives of the phases equal to 0.4, 1.9, 7.1 and 5.1 hr. The initial volume of distribution was 22 ml and the postdistribution volume of distribution was 1076 ml, which indicated an extensive distribution of radioactivity. The apparent blood clearance was 14.7 ml/hr. The radioactivity in the expired air accounted for 51% of the administered dose over the 10-day period. Urinary and fecal radioactivity accounted for 15% and 5% of the administered dose, respectively. The major sites of radioactivity uptake were the liver (up to 22.6% of the dose), kidneys (renal cortex, up to 14% of the dose), bone marrow (up to 14% of the dose), skin (up to 13% of the dose) and skeletal muscle (up to 9% of the dose). Other tissues contained approximately 1% or less of the dose. The overall recovery of radioactivity 10 days postdosing was 95.1 ± 7.5% (mean ± SD.) of the administered single dose. The radioactivity in the blood was almost completely in the plasma during the course of the study. In the plasma, the radioactivity was extensively bound to proteins, as assessed by size-exclusion high-performance liquid chromatography (HPLC). In samples up to 8 hr postdosing. Retention data on size-exclusion HPLC and in vitro incubations using purified proteins suggested that the plasma proteins that bound [^{14}C]2105 were albumin and α_2-macroglobulin. The complex formed between the plasma proteins and [^{14}C]2105derived radioactivity was dissociated on anion-exchange HPLC to indicate that the great majority of plasma radioactivity coeluted with intact [^{14}C]2105 in samples that contained sufficient radioactivity for analysis. There was a timedependent decrease in the proportion of hepatic and renal radioactivity that coeluted with the intact [^{14}C]2105 during the course of the study. The urine did not contain radioactivity that eluted with intact [^{14}C]2105 on anion-exchange HPLC.

Substantial interest in the development of oligonucleotide-based therapeutic agents has been generated (Zamecnik et al., 1978; Stein et al., 1988; Mirabelli et al., 1991; Crooke, 1992). Several first-generation oligonucleotide analogs, in which one or more of the substituents on the internucleotide phosphate are modified, e.g., phosphorothioates, methylphosphonates and phosphorodithioates, have been synthesized and tested (Matsukura et al., 1987; Crooke, 1992). Each of these modifications was shown to enhance the nuclease stability of oligonucleotides significantly (Agrawal and Goodchild, 1987; Crooke, 1991).

Phosphorothioate oligodeoxynucleotides have been studied extensively as potential antisense therapeutic agents. They displayed potent antiviral activities and inhibitory activities against a wide range of mammalian gene products (Mirabelli et al., 1991; Crooke, 1992). Although phosphorothioate oligodeoxynucleotides may display pharmacological activities that are the result of mechanisms other than antisense, typically, non-antisense effects occur at doses significantly greater than the antisense effects (Mirabelli et al., 1991; Crooke, 1992). Optimal antisense activities for phosphorothioate ohgodeoxynucleotides are usually observed with oligonucleotides that are 18 to 21 nucleotides in length (Cohen, 1989).

Phosphorothioate oligodeoxynucleotides have been shown to be stable (half-lives > 24 hr) in serum, cell homogenates, cells, cerebrospinal fluid and organs (Crooke, 1991; Campbell et al., 1990; Agrawal et al., 1988; Crooke, 1993; Stein et al., 1988; Loke et al., 1989; Hoke et al., 1991). They were taken up by many types of cells in tissue culture (for review, see Crooke, 1991; Crooke, 1993) and cellular uptake and in *vivo* activities can sometimes be enhanced by cationic lipids (Bennett et al., 1992; Perlaky et al., 1993).

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; SE-HPLC, size-exclusion HPLC; SAX-HPLC, strong anion-exchange HPLC; ISIS2105, 5'-TTGCTTCCATCTTCCTCGTC-3'; AUC, area under the plasma curve.

Two previous studies investigated the pharmacokinetics of phosphorothioate oligodeoxynucleotides in animals. The pharmacokinetics of a 20-mer phosphorothioate oligodeoxynucleotide were determined after single i.v. or i.p. doses of 30 mg/kg. The oligonucleotide was labeled at each internucleotide linkage with ^{35}S. The compound was shown to be bioavailable after i.p. administration to have broad peripheral tissue distribution and to be cleared primarily by renal excretion. Gel electrophoretic analysis demonstrated significant, albeit slow, metabolism in the liver, kidney and intestines (Agrawal et al., 1991). A 27-mer phosphorothioate deoxyoligonucleotide labeled with ^{35}S was reported to display biexponential elimination in plasma with an elimination half-life in excess of 40 hr after a single i.v. dose of 4.8 mg/kg in the rat (Iversen, 1991).

ISIS 2105 is a phosphorothioate deoxyoligonucleotide that is active against human papillomaviruses (Cowsert et al., 1993). It is currently undergoing pivotal Phase II clinical trials for the treatment of genital warts. There were two objectives of the current study. First, we wanted to develop and evaluate a radiolabeling method that results in higher-specific-activity oligonucleotides to support more detailed pharmacokinetic studies and a more definitive evaluation of metabolism that could be used in clinical trials. Second, we sought to perform more definitive pharmacokinetic distribution and metabolic studies in which metabolites in the plasma, urine and tissues were evaluated using HPLC techniques that support more quantitative analyses.

To achieve these objectives, we radiolabeled ISIS 2105 at the carbon-2 position of all thymidines. Because thymidine is metabolized into CO_2, metabolism can be measured by collecting expired air. This provides an estimate of the total metabolism of the oligonucleotide. Coupled with the extraction of radioactivity and HPLC analysis, a reasonably precise evaluation of metabolism can be achieved. Obviously, a full evaluation of metabolism will require the analysis of intermediates between the intact oligonucleotide and CO_2. We also developed HPLC methods to evaluate the integrity of the radiolabel in tissues and biological fluids.

Materials and Methods

ISIS 2105 Synthesis and Purification

The ^{14}C-labeled phosphorothioate ISIS 2105 was chemically synthesized by using the deoxynucleoside phosphoramidite approach (Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981). The phosphorothioate linkage was generated by oxidizing with ^3H-1,2-benzodithiole-3-one-1,1-dioxide (Iyer et al., 1990) instead of aqueous iodine. All reagents and materials for the solid-phase synthesis of DNA were purchased from commercial sources with the exception of the ^{14}C-thymidine phosphoramidite. The ^{14}C-labeled phosphoramidite synthon was generated from ^{14}C-thymidine (^{14}C at the carbon-2 position of the thymine ring (specific activity, ~56.3 mCi/mmol, Sigma, St. Louis, MO) as described elsewhere (D. Dellinger and H. Sasmor, manuscript in preparation).

The crude synthetic oligonucleotide was purified by trityl-on reverse-phase HPLC by using a methanol gradient in a 0.25 M sodium acetate mobile phase buffer. The HPLC product was acid deprotected and recovered by ethanol precipitation as the sodium salt. The final product was analyzed by using electrophoresis with 20% denaturing polyacrylamide gels and the full length integrity (88% full length material) and the radiochemical purity (88% of counts per minute in the full length product) was determined by laser scanning densitometry and quantitative phosphorimaging (Molecular Dynamics, Foster City, CA), respectively. The specific activity of the final product was ~2.0×10^8 cpm/μmol and had approximately 97.6% phosphorothiodiester content (vs. 2.4% phosphodiester) as determined by high-field nuclear magnetic resonance (500 MHz, University of Missouri, Columbia, MO).

Formulation of ^{14}C-ISIS 2105 in phosphate-buffered saline. We formulated 2.22×10^9 cpm (11.7 pmol) of the purified product by dilution in phosphate-buffered saline, pH 7.0, (Irvine Scientific, Pasadena, CA) to deliver 2×10^7 cpm per 100-μl injection. The solution was sterile filtered through a 0.22-μm cellulose acetate filter (S&S Uniflow, Keene, NH) and the radioactivity was determined in 100 μl by aqueous scintillation.

Animals

Young adult female Sprague-Dawley rats (9-10 weeks old, 175-209 g at the time of randomization) were purchased from Taconic Farms (Germantown, NY). The animals were acclimated to the surroundings of the animal facility used for radioactive studies for approximately 1 week before dosing and were examined by a veterinarian before they were assigned to the study. During acclimation, the rats were housed in individual stainless steel suspended cages with noncontact bedding (Cellu-dri, Shepherd Specialty Papers, Kalamazoo, MI). Twenty-four hours before dosing, the rats to be used for urine, feces and expired air collection were transferred to Nalgene metabolism cages (Nalge, Rochester, NY). At the time of dosing, the animals used for the collection of urine, feces and expired air were transferred to glass metabolism cages (Vanguard International, Neptune, NJ). The animals used for the collection of retro-orbital blood samples, but not for urine and feces, were housed individually in stainless steel suspended cages. Each cage was labeled with the animal identification number. Food and water were allowed *ad libitum*. The food consisted of Purina Standard Rodent Chow (#5001, Raltech Scientific Services, St. Louis, MO) in pellet form. The water was city tap water.

Compound Administration

The [^{14}C]2105 was administered in solution in phosphate buffer, pH 7, to rats by i.v. injection (100 μl) into the caudal tail vein. The concentration of [^{14}C]2105 was 1.2 mM; therefore, the dose level was approximately 3.6 mg/kg. The actual doses administered were calculated by using the assayed concentration of radioactivity in a 100-μl volume of the formulation. The [^{14}C]2105 was administered to 25 female rats. Five rats were used for the collection of urine, feces, expired air and tissues at the time of sacrifice. The remaining 20 animals were used for the collection of blood at intervals after dosing and tissues at the time of sacrifice.

Sample Collection

Urine and feces. Urine and feces were collected from the five animals housed in glass metabolism cages at 0 to 4, 4 to 8, 8 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, 144 to 168, 168 to 192, 192 to 216 and 216 to 240 hr after administration of [^{14}C]2105.

The urine and feces were frozen immediately on excretion and were kept frozen for the entire collection period. Before sacrifice, the animals were made to urinate by gentle pressure on the urinary bladder and this urine was combined with the last sample collected. A thorough cage wash with water was performed at the time of sacrifice. At sacrifice, the blood and plasma were obtained as described subsequently. Urine, feces and cage washings were stored in a -20°C freezer.

Expired air. The radioactivity in the expired air was collected from the five animals housed in glass metabolism cages in a series of two traps. The traps contained 6 M KOH for the collection of $^{14}CO_2$. The radioactivity in the expired air was collected during the following intervals: 0 to 4, 4 to 8, 8 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, 144 to 168, 168 to 192, 192 to 216 and 216 to 240 hr after the administration of [^{14}C]2105. The KOH samples were stored in a 4°C refrigerator until analysis.

Blood. Single blood samples (approximately 50–100 μl each) were obtained by retro-orbital puncture from two animals at each of the following time points: 0.5, 1.5, 3, 6, 12, 36, 56, 80, 104 and 128 hr. Capillary tubes containing blood were stored on ice until the blood was

aliquoted into combustion cones. Two animals were sacrificed at the following time points: 1, 2, 4, 8, 24, 48, 72, 96, 120 and 144 hr after dosing. At sacrifice, the animals were anesthetized with CO_2 and the blood (5 ml) was recovered by cardiac puncture and transferred into heparinized Vacutainer tubes (Becton Dickinson, Rutherford, NJ). The plasma was obtained from the blood by centrifugation and kept on ice before storage at -20°C. Animals were sacrificed by exsanguination.

Tissue. At the time of sacrifice, the liver, kidneys, spleen, lungs, brain, eyes, bone (femur), skeletal muscle (representative sample), ovaries, uterus, representative dorsal skin and carcass were collected and immediately frozen on dry ice and stored in a -20°C freezer.

Analytical Procedures

All determinations of total radioactivity in the tissues, excreta and blood were made with a Beckman LS 6000 scintillation system (Fullerton, CA).

The weight of the urine was determined. The radioactivity in duplicate samples of urine, plasma and cage rinse was determined in Scint-A XF (Packard Instrument, Downers Grove, IL). The radioactivity in duplicate samples of KOH was determined in modified Bray's solution (Nomeir et al., 1992). Feces were weighed and homogenized with distilled water (20% w/w) with a Brinkmann Polytron homogenizer (Lucerne, Switzerland).

The liver, lung, brain and spleen samples were weighed and finely minced before combustion. The skeletal muscle (representative sample), blood, uterus, eyes and ovaries were weighed but not pretreated before combustion. The bone marrow was removed from bone and weighed before combustion. The renal medulla and cortex were excised from the kidneys and aliquots were weighed before combustion. The carcasses from the animals that were used to determine mass balance and the skin (representative sample) were weighed, powdered on dry ice with a Waring (New Hartford, CT) blender and homogenized with distilled water (33% w/w) with a Brinkmann Polytron homogenizer.

Duplicate samples (total sample size allowing) of fecal homogenates, tissue minces and whole tissues were aliquoted and then underwent combustion with a Packard Tricarb oxidizer, model 307. The ^{14}C radioactivity was trapped in Carbosorb II (Packard Instrument). The recovery of the combustion system was determined on a daily basis and ranged from 97% to 100%.

Extraction of tissue radioactivity. To approximately 1 g of liver, 0.5 ml of extraction buffer (0.5% NP-40, 20 mM Tris HCl, pH 8.0, 20 mM EDTA, 100 mM NaCl and 2 mg/ml of proteinase K) was added and the tissue was homogenized using a Bessman tissue pulverizer (Spectrum, Houston, TX) followed by using the A pestle of a 7-ml Dounce-type tissue grinder (Wheaton, Millville, NJ). One kidney from each animal was dissected into the cortex and medullar regions. We combined 0.1 to 0.2 g with 0.25 ml of extraction buffer and homogenized it in a 1-ml Dounce-type tissue grinder (Wheaton) by using the A pestle. All samples were then incubated at 65°C for 24 hr, followed by centrifugation at 16,000 × g at 4°C for 15 min. The supernatants were removed and stored frozen at −70°C until analysis.

All samples were filtered through an Ultrafree-MC 0.22-μm filter (Millipore, Bedford, MA) at 4°C. Before analysis, the liver and kidney samples were thawed and unlabeled ISIS 2105 was added to a final concentration of 100 μM. The plasma samples were thawed at room temperature and immediately analyzed.

HPLC. SAX-HPLC was used to determine the metabolic profile of the plasma and urine and hepatic and renal tissues. Ion-exchange analyses were carried out using a Beckman System Gold liquid chromatography system with model 126 pumps, model 507 autoinjector, and model 166 detector. We analyzed 40 μl of each sample of plasma and urine, or of liver or kidney homogenate, at 260 nm on a 4.6 × 100-mm Gen Pak Fax column (Waters, Milford, MA) by using the following buffers and gradient: buffer A, 0.086 M Tris HCl, pH 8.0, 20% methanol; buffer B, 0.086 M Tris HCl, pH 8.0, 1.5 M NaBR; gradient, 0% B isocratic for 5 min and then linear to 60% B over 45 min at a flow of 0.5 ml/min. Fractions (0.5 ml) were collected and added to 5 ml of Readysafe scintillation cocktail (Beckman) and then counted in a Beckman model LS6000IC scintillation counter.

The plasma samples were also analyzed by SE-HPLC by using a Hewlett-Packard (Pasadena, CA) model 1090 M liquid chromatography system and a 7.8 × 300-mm TSK-gel G2000 SWXL column (Tosohaas, Montgomeryville, PA). The analytes were carried out in 0.05 M Na_2HPO_4, 0.1 M Na_2SO_4, and 0.05 M NaH_2PO_4, pH 7.0, at a flow of 0.45 ml/min. On-line radiochemical detection was accomplished with a Radiomatic FLO-ONE/beta model A-525A detector (Packard Instruments, Meriden, CT). The scintillation cocktail, Ultimate-Flo-V, was purchased from Packard Instruments and was used at a flow rate of 0.9 ml/min.

Calculations

The concentrations of radioactivity in the samples processed by combustion were corrected for the recovery efficiency of the combustion system, which was determined daily before the combustion of experimental samples. The observed radioactivity values were converted to compound radioequivalent concentrations. The radioequivalents were defined as the amount of parent compound, at the specific activity as administered, that would result in the observed disintegrations per minute value. Compound equivalents in a biological sample were determined by dividing the disintegrations per minute in the sample by the specific activity of the compound in disintegrations per minute per microgram. The compound equivalents were expressed in micrograms per gram of tissue and, when possible, as a percentage of the administered dose/organ or tissue. For the purpose of calculating a mean ± S.D., the tissue samples in which the radioactivity was less than twice the background for the system, the equivalents were less than 0.005 microgram equivalents per gram or the radioactivity was less than 0.005% of the dose were considered to have a value of zero.

The radioactivity in urine, feces, volatile traps and cage rinse was expressed as a percentage of the administered dose for each time interval and as a cumulative percentage. For the purposes of calculating a mean ± S.D., urine, feces, expired air and cage wash samples in which the radioactivity was less than twice the background for the system or the radioactivity was less than 0.05% of the dose were considered to have a value of zero.

Pharmacokinetic parameters for the ISIS 2105 equivalents in blood and plasma were calculated by polyexponential curve fitting of the observed concentrations, using the RSTRIP, Polyexponential Curve Fitting Program, Version 4.02 (Micromath Scientific Software, Salt Lake City, UT). The areas under the concentration-time curve and the terminal elimination half-lives for tissues were calculated by using noncompartmental analysis of the observed data (Shumaker, 1986).

Results

Percentage of the Dose of [^{14}C]2105 in Tissues

The percentage of the dose in the tissues at intervals after the single-dose administration is summarized in table 1. The maximal percentage of the dose in the liver (23%) was observed 4 hr after dosing. The maximal percentages in the renal cortex (14%) and renal medulla (3%) were observed at 4 and 8 hr after dosing, respectively. At 240 hr after a single i.v. administration, a significant portion of the radioactivity remained in the tissues. The highest percentage of the dose was observed in the renal cortex (4%), followed by the skin and bone marrow (3% each). A lesser percentage was observed in the liver (2%). All other tissues contained less than 1% of the dose. The total percentage of the dose recovered in the tissues and carcass at 240 hr was approximately 20% (table 2).

Urinary Excretion of [^{14}C]2105-Derived Radioactivity

The mean cumulative percent of [^{14}C]2105-derived radioactivity excreted in the urine is summarized in figure 1. The

TABLE 1

Percentage of dose in tissues[a] of female Sprague-Dawley rats at intervals after a single i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg

Time	Liver	Renal Medulla[b]	Renal Cortex[b]	Lung	Skeletal Muscle[c]	Bone Marrow[d]	Skin[c]
				Fraction of dose (%)			
hr							
1	16	1	7	1	9	7	9
2	20	1	10	—[f]	7	6	13
4	23	2	14	—	5	10	12
8	19	3	12	—	2	12	6
14	14	1	13	—	1	14	9
48	10	2	10	—	2	13	2
72	8	2	10	—	—	10	11
96	6	1	8			8	8
120		1	7			8	4
144	3	1	6	—	—	5	6
240[e]	2	—	4			3	3

[a] Tissues containing ≥ 1% dose are included. Brain, spleen, ovaries, uterus and eyes contained < 1% dose at any given time.
[b] The percentages were calculated by assuming that renal cortex = 69% and renal medulla = 31% of the total kidney weight (the percentages were generated from kidney dissections in ADL MAP laboratory).
[c] The percentages were calculated from the organ weights and by assuming that muscle = 50% of the body weight and skin = 11% of the body weight (Burka et al., 1987).
[d] The percentages were calculated by assuming that bone marrow = 3% of the total body weight (Baker et al., 1979).
[e] All percentages are average data from two animals except for time 240 hr, which is the mean data from five animals.
[f] Contained < 1% of dose.

TABLE 2

Recovery of drug-related radioactivity from female Sprague-Dawley rats 246 hr after a single i.v. administration of [^{14}C]2105 at a dose of approximately 3.6 mg/kg

Sample	Animal No.					Mean ± S.D.
	2921	2922	2923	2924	2925	
	Dose recovered (%)					
Urine	4.5	16.2	15.3	15.8	4.1	15.1 ± 2.2
Feces	51.8	4.2	4.3	5.9	43.7	4.6 ± 0.7
Expired air		51.6	56.1	50.4	....	51.1 ± 5.1
Tissues	16.1	21.6	16.3	22.9	20.5	20.3 ± 2.2
Cage wash	3.6	4.0	4.8	5.0	2.3	4.0 ± 1.1
Total	95.7	97.6	100.6	99.2	62.1	95.1 ± 7.5

percentage of [^{14}C]2105-derived radioactivity excreted by the urinary route was 15.1 ± 2.2%, primarily within the first 72 hr. The urinary excretion rate resulted in an elimination half-life of 55 hr (table 3).

Fecal Excretion of [^{14}C]2105-Derived Radioactivity

The mean cumulative percent of [^{14}C]2105-derived radioactivity eliminated in the feces is summarized in figure 1. The percentage of [^{14}C]2105-derived radioactivity excreted in the feces was 4.6 ± 0.7%, primarily within the first 96 hr.

Excretion of [^{14}C]2105-Derived Radioactivity in Expired Air

The excretion of [^{14}C]2105-derived radioactivity in expired air is illustrated in figure 1. The majority of the i.v. dose of [^{14}C]2105 (51.1 ± 5.1% of the dose) was eliminated by expired air, primarily within the first 96 hr. The expiration rate of [^{14}C] 2105-derived radioactivity resulted in an elimination half-life of 60 hr (table 3).

Total Recovery of [^{14}C]2105-Derived Radioactivity

The total recovery of [^{14}C]2105-derived radioactivity after a single i.v. dose of [^{14}C]2105 at 3.6 mg/kg is summarized in table 2. The majority (51%) was recovered in the expired air. A smaller percentage was recovered in the urine (15%) and in the feces (4.6%). The remaining radioactivity was recovered in the tissues and carcass (20%) and the cage wash (4%). Overall recoveries ranged from 82% to 101% with a mean for the five animals of 95.1 ± 7.5%.

Phannacokinetics of ISIS 2105 Equivalents in Blood and Tissues

The pharmacokinetic data analysis is based on microgram equivalents of ISIS 2105 present in the matrices. As such, the data describe the pharmacokinetic behavior of ISIS 2105-related radioactivity and not necessarily unchanged parent ISIS 2105.

Pharmacokinetics in blood. After i.v. administration, a peak blood radioactivity concentration of 17.2 μg equivalents/g was achieved. The concentration versus time profile in the blood is shown in figure 2, along with the fitted polyexponential curve used to calculate pharmacokinetic parameters. The blood radioactivity versus time profile was polyexponential, with four phases (table 3). The initial phase had a half-life of 0.4 hr and the terminal elimination phase had a half-life of 51 hr. The plasma data paralleled the blood elimination profile but the terminal elimination half-life was 40 hr (data not shown). At each of the corresponding time points, all or most of the radioactivity was associated with plasma and not with formed elements of blood. These data strongly suggest no binding or distribution of ISIS 2105 on or in the red blood cells. As would be expected from the long terminal half-life, the apparent blood clearance after i.v. administration was low, i.e., 14.7 ml/hr (table 3).

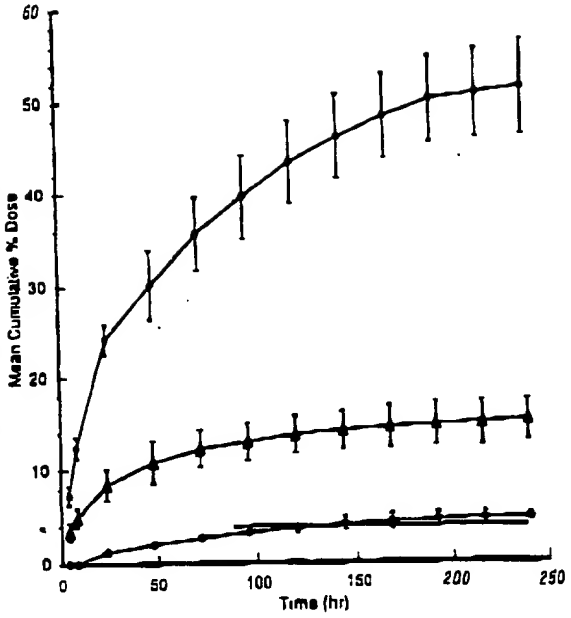

Fig. 1. Mean (± S.D., n = 4) cumulative percentage of the dose eliminated as $^{14}CO_2$ (■), in urine (▲) and in feces (◆) of female Sprague-Dawley rats after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg.

The initial volume of distribution was 22.0 ml and the postdistribution volume of distribution was 1076 ml, which indicates extensive partitioning into the tissues.

Pharmacokinetics of tissue radioactivity. The tissue radioactivity concentrations were highest in the liver, kidney, spleen and bone marrow (table 1). The elimination half-life from the liver was 62 hr, which approximated that seen in the blood. The elimination half-life was prolonged in the kidney, with observed values of 112 hr in the cortex and 156 hr in the medulla. The elimination half-life of radioactivity from the bone marrow was 78 hr (table 3).

The areas under the concentration versus time curves for the tissues (bone marrow, liver, spleen and kidney) were calculated to gauge a relative drug exposure level after i.v. administration of [^{14}C]2105. For these four tissues, comparisons were made per gram of tissue and not adjusted for the total organ weights. Of these tissues, the liver and spleen received the lowest exposure, with $AUC_{(0-\infty)}$s of 1158 and 1618 μg equivalents-hr/g, respectively. The kidney medulla received 5.6 times the liver exposure or 6497 μg equivalents-hr/g. The kidney cortex received the highest exposure, 15688 μg equivalents&/g, approximately 2.4 times the medulla and 13.5 times the liver exposure. The bone marrow exposure was approximately 2 times the liver exposure, with an AUC of 2526 μg equivalents-hr/g. These AUCs were much higher than the blood AUC; this again suggested significant partitioning of ISIS 2105 equivalents into the tissues.

Characterization of Plasma, Tissue and Urinary Radioactivity

Plasma radioactivity was present in two peaks as assessed by SE-HPLC (fig. 3). The majority of the radioactivity eluted with a retention time that was the same as that of the known complex formed between purified rat albumin and [^{14}C]2105 (approximately 18 min). A smaller proportion of the plasma radioactivity eluted with a retention time that was the same as that of the known complex formed between purified human α_2-macroglobulin and [^{14}C]2105 (approximately, 15 min). Little or no radioactivity eluted with a retention time of authentic [^{14}C] 2105 (approximately 23 min). Preliminary studies (Cossum et al., manuscript in preparation) demonstrated that the affinity of ISIS 2105 for albumin and α_2-macroglobulin was in the micromolar range and that binding to both proteins was saturated when ISIS 2105 concentrations exceeded 5 to 10 μM.

To determine the integrity of ISIS 2105, plasma was applied directly to SAX-HPLC. The buffer in that system results in the extraction of radioactivity from plasma proteins. When a tissue pulverizer step was included, the total recovery of the radioactivity was approximately 60%. If only simple homogenization with a Dounce homogenizer was used, only approximately 30% of the total radioactivity was recovered. However, a comparison of the samples by HPLC revealed no differences; thus, both methods probably extract representative samples from the tissues. When plasma sampled from rats for up to 8 hr postdosing was subjected to SAX-HPLC, the majority of the radioactivity eluted with authentic [^{14}C]2105 (fig. 4). The radioactivity in peaks eluting earlier than authentic [^{14}C]2105 were, presumably, shorter metabolites of [^{14}C]2105. At the 8-hr time point, approximately 38% of the radioactivity represented intact ISIS 2105. A determination of the proportion of intact ISIS 2105 in the plasma obtained after 8 hr (*i.e.*, ≥ *24* hr) was not possible because of the low levels of radioactivity in those samples.

Figures 5 and 6 show anion-exchange radiochromatograms of extracts of hepatic and renal cortex tissues, respectively,

TABLE 3
Pharmacokinetic parameters of ISIS 2105 equivalents after a single i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg

Sample	AUC	Distribution $T_{1/2}$	Terminal $T_{1/2}$	C_{max}	T_{max}	Apparent Clearance	V_c*	V_{ss}*
	μg equivalents-hr/g	hr	hr	μg equivalents/g	hr	ml/hr	ml	ml
Blood	52	7.1	51	17.2	0.5	14.7	22.0	1076
Urine	—		55	—	—			
Expired air	—		80	—	—			
Liver	1158		62	19.0	4			
Renal medulla	6497		156	42.7	8			
Renal cortex	15,688		112	87.4	4			
Spleen	1618		163	18.6	1			
Bone marrow	2526		78	18.1	24			

* Initial volume of distribution.
* Postdistribution volume of distribution.

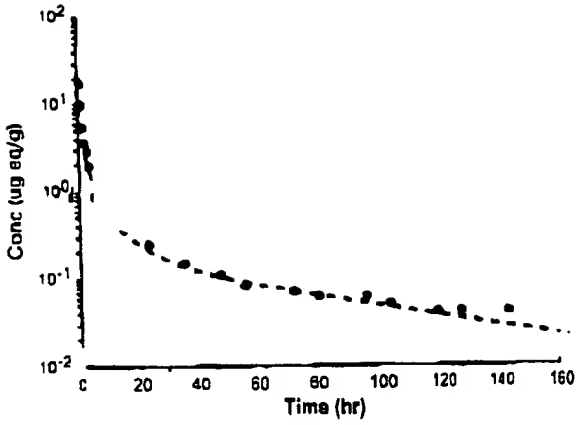

Fig. 2. Concentration of ISIS 2105 equivalents in the blood of female Sprague-Dawley rats after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Each point represents the average of the values for two animals. The dashed line represents the line of best fit estimated from the four-compartment model.

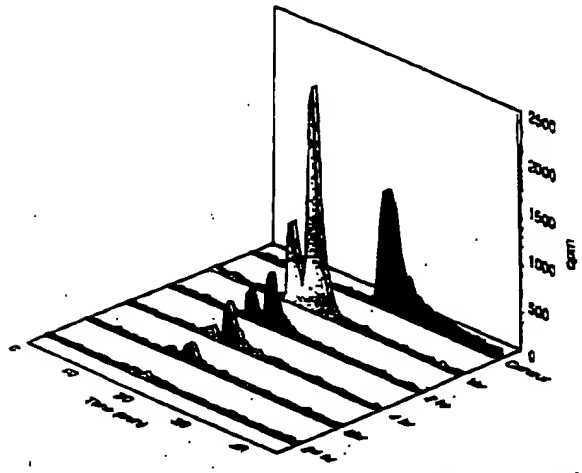

Fig. 3. SE-HPLC radiochromatograms of plasma sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C] 2105 at a dose of 3.6 mg/kg. The control peak represents [^{14}C]2105 that has a retention time of approximately 23 min. The plasma samples from dosed rats contained radioactive peaks eluting at approximately 15 min and approximately 16 min.

sampled at various times after the administration of [^{14}C]2105. The proportion of apparently unchanged [^{14}C]2105 decreased with time. The proportion of radioactivity present as apparently intact [^{14}C]2105 varied between the liver and kidney; [^{14}C]2105 was more stable in the kidney than in the liver. After 24 hr, approximately 15% of the radioactivity extracted from the liver represented intact ISIS 2105 and only trace levels of intact ISIS 2105 were present at 48 hr. By contrast, even after 96 hr, 48% of the radioactivity extracted from the kidney represented intact ISIS 2105.

Figure 7 shows anion-exchange radiochromatograms of urine sampled for up to 96 hr postdosing. Little or no radioactivity eluted with the same retention time as authentic [^{14}C]2105 at any sampling time.

Discussion

The radioactivity in the blood was located almost entirely in the plasma for 3 to 4 days after dosing. Only low levels of radiolabel were detected associated with the cellular components of the blood. The radioactivity in the plasma was associated with albumin and α_2-macroglobulin; only a trace of free [^{14}C]2105 was detectable in the plasma. Preliminary data suggest that ISIS 2105 binds to these proteins with a relatively low affinity and that binding is clearly saturable (Cossum et al., manuscript in preparation). The radioactivity bound to those plasma proteins represented mostly intact [^{14}C]2105. However, the levels of radioactivity in plasma were insufficient to determine the integrity of the drug at times beyond 8 hr postdosing.

There was a rapid and substantial distribution of radioactivity from the blood into the tissues. The initial volume of distribution of *22.0* ml approximates the blood volume of the rats used in this study. The postdistribution volume of distribution was 1076 ml, a value that indicated the distribution of radioactivity into a "deep" compartment. A four-compartment model fit the data best *(i.e., r^2 0.998 vs. 0.823* for a two-compartment model). However, in other studies we have performed, a two-compartment model fit the data best. Consequently, we think the complexity of the model most likely results from minor animal-to-animal variations and the fact that radioequivalents were considered rather than intact drug. In future studies, we will attempt to address this issue in more detail. In any event, the elimination half-life was prolonged and examination of intact drug levels suggested a relatively prolonged elimination half-life for the intact drug and radioequivalents. The primary organs of accumulation of radioactivity were the liver, kidneys (particularly the renal cortex), bone marrow and spleen. The kinetics of distribution of the radiolabel into the peripheral organs varied. Peak levels were achieved in the liver and kidney 4 hr after the dose. By contrast, peak levels in the skeletal muscle were observed 1 hr after the dose and peak levels in the bone *marrow* were not achieved until 24 hr postdose. Skin accumulated a surprising amount of radiolabel, with peak levels that occurred 2 to 4 hours after administration.

That the radioactivity in various organs represented intact [^{14}C]2105 and metabolites was demonstrated by extraction followed by SAX-HPLC. Although its metabolism in the liver was extensive, the rate of metabolism was relatively slow. Twenty-four hours postdosing, approximately 15% of the total hepatic radioactivity was present as intact [^{14}C]2105. The metabolism in the kidney was minimum because intact drug was present even 96 hr postdosing. The extensive metabolism in the liver, coupled with the lack of metabolites found in the kidney and complete absence of intact drug in the urine, suggested that, after the initial distribution, only limited redistribution between the liver and kidney (and presumably other organs) occurred.

In this study, "C-labeled ISIS 2105 was synthesized using "C-labeled thymidine labeled at the carbon-2 position of thymine. The fate of thymidine is either through utilization into DNA *or* degradation to thymine (Henderson and Patterson, 1973). In mammals, the carbon-2 position carbon of thymine is degraded to CO_2 and so production of $^{14}CO_2$ would be expected when thymine is labeled at that carbon (Henderson and Patterson, 1973). Thymine could have been generated from ISIS 2105 in vivo in at least two ways. The putative metabolic scheme would involve hydrolysis of the phosphorothioate backbone, which eventually would generate thymidine. Subsequently, thymidine phosphorylase could dethiophosphorylate the thymi-

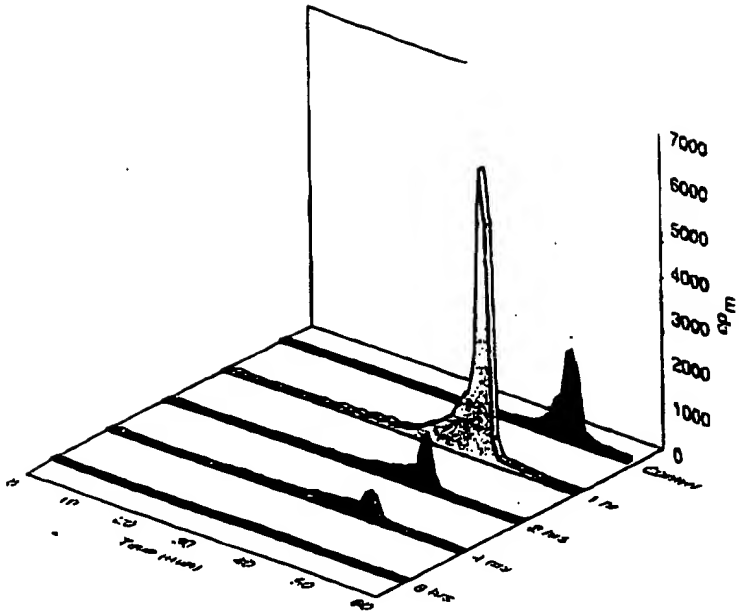

Fig. 4. SAX-HPLC radiochromatograms of plasma sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. The plasma contained radioactivity that eluted principally with [^{14}C]2105 (approximately 43 min).

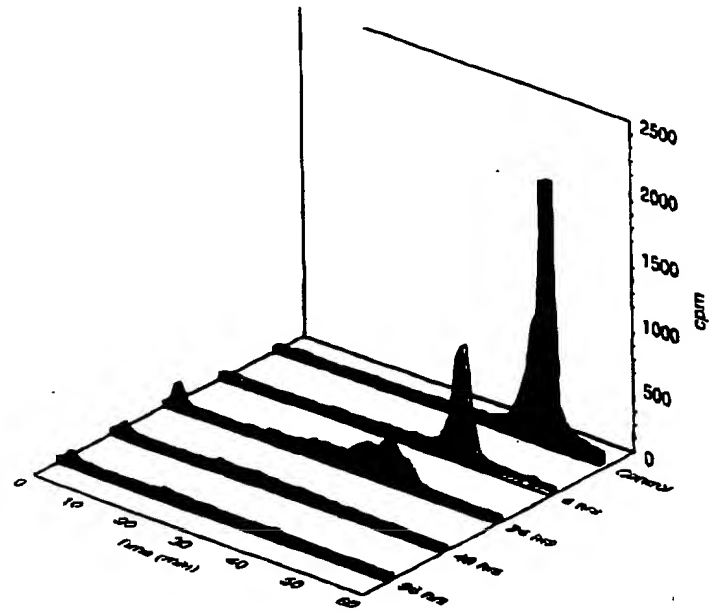

Fig. 5. SAX-HPLC radiochromatograms of extracts of liver sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Authentic [^{14}C]2105 elutes at approximately 43 min.

dine or the thiophosphate might be oxidized first and the phosphorylase then would release phosphate. Thymine could then be further metabolized to CO_2. Thymidine phosphorylase is a cytoplasmic enzyme with highest activity in the intestinal mucosa, liver, bone marrow, kidney and spleen (Friedkin and Roberts, 1954). Consequently, the limited amounts of degradation products in the kidney suggest that the rate-limiting step in the pathway is hydrolysis. This is consistent also with the slow overall metabolism observed. In this regard, it is important also to recognize that approximately 2.4% of the internucleotide linkages in [^{14}C]2105 were phosphate resulting from the oxidation of the phosphorothioate during synthesis. This is greater than the routine specification for unlabeled ISIS 2105 (0.6%) and could account for a slightly greater hydrolytic rate of radiolabeled ISIS 2105.

Alternatively, it is possible that thymine was removed from [^{14}C]2105 by a glycosidic bond cleavage without prior hydrolysis of the internucleotide linkage. DNA glycosylases are nuclear enzymes that remove purines or pyrimidines from DNA as part of repair mechanisms. The resulting apurinic or apyrimidinic site in the oligonucleotide would be expected to be susceptible to endonuclease action (Warner, 1983). Further studies are required to elucidate the mechanism(s) of ISIS 2105 degradation.

The principal, albeit slow, mechanism of clearance of ISIS 2105 in rats is metabolism. Of the total dose, in excess of 50%

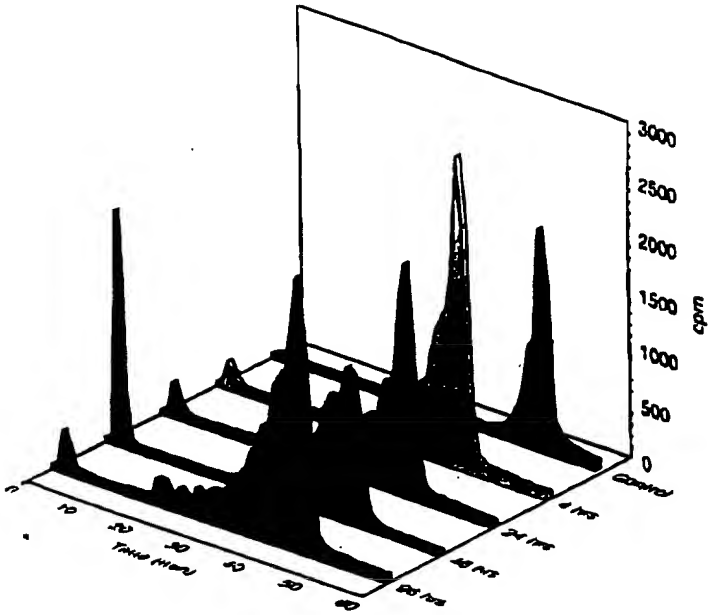

Fig. 6. SAX-HPLC radiochromatograms of extracts of renal cortex sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Authentic [^{14}C]2105 elutes at approximately 43 min.

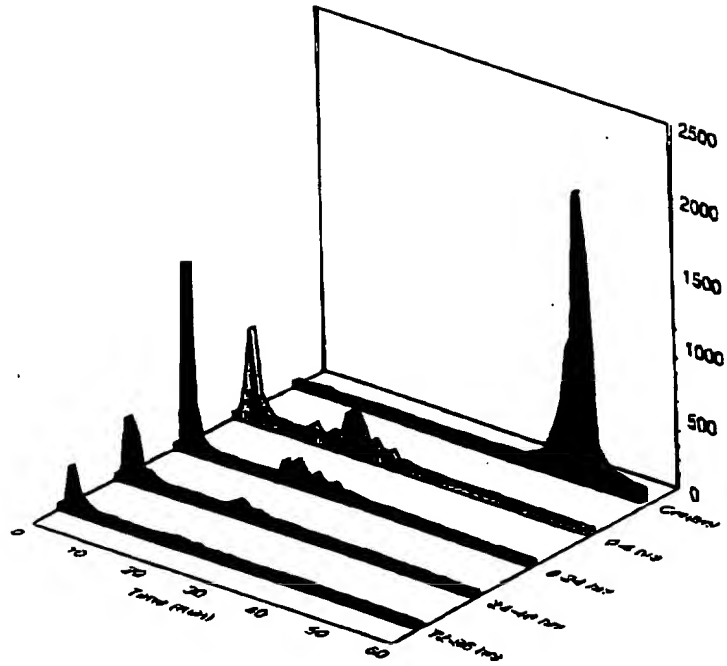

Fig. 7. SAX-HPLC radiochromatograms of urine sampled from female Sprague-Dawley rats at various times after i.v. administration of [^{14}C]2105 at a dose of 3.6 mg/kg. Authentic [^{14}C]2105 elutes at approximately 43 min.

was recovered in expired air. Only metabolites were found in the urine and urinary excretion accounted for only 15% of the total dose. There was no evidence of significant biliary secretion or hepatobiliary recirculation.

Although the results of this study and the study of Agrawal et al. (1991) were similar, there were several important differences. Agrawal et al. (1991) did not report on plasma protein binding. ISIS 2105, and every other phosphorothioate oligonucleotide we have studied, binds extensively to plasma proteins. The binding is low affinity and high capacity, which is traditionally associated with many other classes of drugs and their interaction with plasma proteins. We consider this to be one of the principal reasons that phosphorothioate oligonucleotides are not cleared rapidly by renal filtration. At the 30-mg/kg dose used by Agrawal et al. (1991), we would expect the plasma protein binding to be saturated and, therefore, to result in significant levels of free drug in the plasma. Approximately 30% of the i.v. or i.p. dose of radioactivity after dosing with the ^{35}S-labeled oligonucleotide was recovered in the urine by 24 hr postdosing and gel electrophoresis of the urine indicated the presence of intact drug. At early times (0–6 hr) after i.v. dosing, 95% of the urinary radioactivity cocluted with intact drug and,

up to 24 hr postdosing, only 15% degradation was noted. The urine of mice dosed i.p. contained material that was only 10% intact parent drug at 24 hr postdosing. At no time did we observe intact drug in the urine.

Two preliminary reports of studies on an anti-rev 27-mer phosphorothioate oligodeoxynucleotide have been presented (Bigelow et al., 1991; Bigelow et al., 1992). In these studies, the drug was given by a variety of routes (i.v. bolus, i.v. infusion, s.c. and p.o.) and concentrations of intact drug were determined by HPLC separation and ultraviolet detection. Excellent bioavailability from s.c. sites and limited p.o. bioavailability were reported. Tissue accumulation similar to our results was reported. A significantly shorter plasma half-life was reported but this was probably the result of the relatively insensitive detection methods. We have studied the pharmacokinetics of several oligonucleotides after intradermal, i.m., i.p. and intravitreal administration and articles describing these results are in preparation.

Agrawal et *al.* (1991) reported that 85% to 90% of the radioactivity present in most tissues of mice 48 hr after the administration of a ^{35}S-labeled phosphorothioate oligonucleotide was associated with intact drug. However, only 50% of the radioactivity in the liver and kidneys was associated with the parent drug at 48 hr. Only approximately 5% of hepatic radioactivity was present as apparently unchanged [^{14}C]2105. By contrast, the majority of renal cortex radioactivity in 96-hr samples eluted with authentic [^{14}C]2105. Larger molecular weight bands were found when tissue extracts were analyzed by polyacrylamide gel electrophoresis in the study of Agrawal et *al.* (1991). They speculated that the radioactivity might represent longer oligonucleotides. We did not observe any evidence of these species. An alternative explanation for these observations might be that the material represented drug bound to α_2-macroglobulin, a protein which is found in mice (LaMarre et *al.*, 1991).

All these differences might be explained by variations between species or differences caused by different sequences. Moreover, Iverson (1991) reported that, at lower doses, an anti-*rev* oligonucleotide was excreted intact in urine, which further suggests that there may be sequence differences. However, in studies in our laboratories, we have not observed such significant differences as a function of species or the specific sequence of the drug. Additional studies are clearly indicated.

In summary, after i.v. administration of [^{14}C]2105 to rats, the tissue distribution of radioactivity was extensive and the radioactivity was eliminated slowly. Although the drug was apparently extensively metabolized, the available evidence shows that the rate of metabolism was relatively slow. Metabolic studies were facilitated by synthesizing an oligonucleotide containing ^{14}C-labeled thymidine. Circulating radioactivity was extensively bound to plasma proteins, a phenomenon that may retard the renal filtration of unchanged drug. Finally, we observed binding of ISIS 2105 to α_2-macroglobulin. In addition to providing a plasma reservoir of ISIS 2105, biding to α_2-macroglobulin could be important in the pharmacokinetics of ISIS 2105 because this protein has been shown to be taken up by various cells through a receptor-mediated mechanism (James, 1990).

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6
Pharmacokinetic Properties in Animals

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I. INTRODUCTION

Antisense technology is a fundamentally different approach to disease treatment both because it provides a simple and rational approach to drug discovery and because it targets a new biological template, mRNA. The mRNA target represents an earlier and, by definition of the antisense method, a more selective target for interrupting the translation of proteins that are involved in the cause or maintenance of disease.

The unique pharmacology of antisense oligonucleotides requires intense study of their in vivo pharmacokinetics. In addition, to aid in the characterization of the safety of antisense oligonucleotides, their exposure as a function of dose and ultimately the rates and mechanisms of clearance from the body must be defined. The development of these compounds as therapeutics has received increasing attention in recent years. As is often the case with new therapeutics and chemistries, the lack of sensitive and selective bioanalytical methods precludes the use of unlabeled materials. Therefore, many of the studies characterizing animal and human pharmacokinetics have relied on radiolabel tracer experiments. Over the past few years, however, methods that provide selective, sensitive, and reliable quantitation of oligonucleotides in biological fluids (1–6) and tissues (7) have allowed characterization of unlabeled material and provided information on metabolism of these compounds. These methods have greatly facilitated the advance of our understanding of antisense phosphorothioate oligonucleotide pharmacokinetics. The objectives of this review are to provide a summary of more recent progress in animal pharmacokinetics and to provide an integrated understanding of the absorption, distribution, metabolism, and excretion of phosphoro-

thioate oligodeoxynucleotides (PS ODNs) in animals. Ultimately, pharmacokinetics of antisense oligonucleotides must include assessment of the kinetics of oligonucleotide delivery and clearance from the site of action coupled with ultimate pharmacological activity, antisense inhibition of specific target mRNA. This chapter will begin to address these issues by providing a review of the pharmacodynamics in the context of whole-animal pharmacokinetics to include tissue distribution and clearance kinetics of parent antisense oligonucleotide.

II. SEQUENCE-INDEPENDENT PHARMACOKINETICS

The existing data demonstrate that phosphorothioate oligodeoxynucleotide (PS oligonucleotide) pharmacokinetics are generally independent of sequence (8,9), suggesting that data from one sequence enhances our understanding of the pharmacokinetic characterization of other PS oligonucleotides. Antisense PS oligonucleotides are generally made up of 18–24 nucleotides linked with 17–23 phosphorothioate linkages each with a net negative charge. Considering their length, the number of negative charges, and their hydrophilicity, it is not surprising that there is little effect of sequence or the order of the nucleotides on the physical/chemical properties of these compounds. Given these similar properties, it should not be surprising that PS oligonucleotides as a class share many properties including pharmacokinetic properties. This hypothesis is supported by empirical evidence demonstrating that there are many similarities in pharmacokinetic properties of different oligonucleotide sequences. For example, monkeys infused with 1 mg/kg over 2 h with any of four different oligonucleotide sequences all have similar C_{max} values (Fig. 1).

The rates of plasma clearance of the oligonucleotides are also similar from sequence to sequence as can be seen from the similar slopes in Fig. 1. AUC values obtained in monkey studies also appear to be similar between sequences and then across species supporting the concept of sequence independence of the pharmacokinetics of these compounds (Table 1). Sequence-independent pharmacokinetics has been observed for a number of sequences in all of the animal species examined including human.

Tissue distribution also appears to be largely independent of sequence in mouse and monkey (Fig. 2). Kidney and liver always exhibit highest concentrations of PS oligonucleotides followed by spleen and lymph nodes. While subtle differences in tissue distribution have been observed in closely controlled studies presumably due to sequence-dependent differences in protein binding (10) or nuclease activity, these differences were not great enough to alter the relative order of distribution organs nor was there any measurable effect on plasma pharmacokinetics. Finally, mass balance excretion of two different sequences of radiolabeled PS oligonucleotide exhibited nearly equivalent excretion profiles 10 days after single-dose administration (Fig. 3).

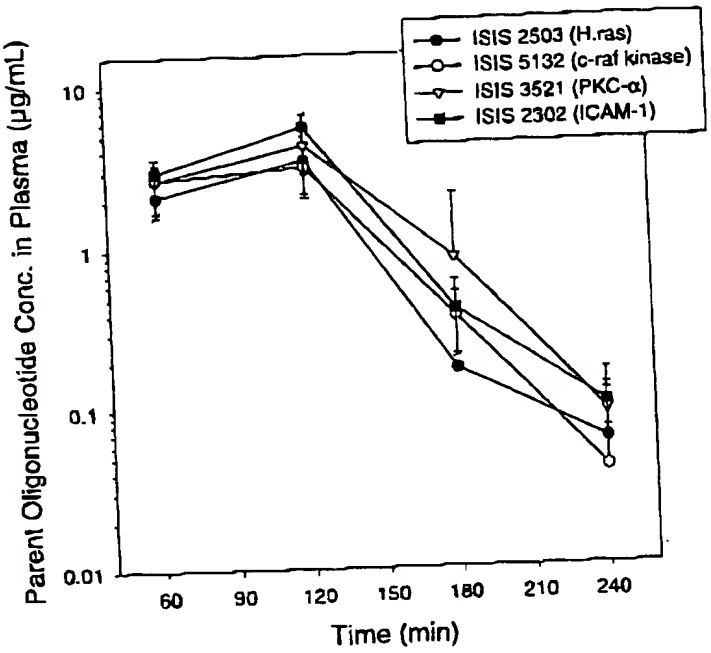

Figure 1 Plasma concentrations of intact oligonucleotides during and following a 2-h intravenous infusion of 1 mg/kg of various oligonucleotides. Plasma samples were extracted and analyzed by CGE. The data are expressed as the intact parent oliogonucleotides (each data point represents the average of three to five monkeys).

Table 1 Comparison of Monkey and Human Pharmacokinetic Parameters

Compound	Species	C_{max} (μg/mL)	AUC (μg/min/mL)
ISIS 2302	Monkey	4.59 ± 0.16	580 ± 11
	Human	3.96 ± 1.02	506 ± 56
ISIS 5132	Monkey	3.27 ± 0.89	396 ± 85
	Human	5.96 ± 2.85	720 ± 243
ISIS 3521	Monkey	4.30 ± 0.75	486 ± 63
	Human	5.45 ± 1.94	704 ± 158

Summary of maximum concentration in plasma (C_{max}) and area under plasma concentration-time curve (AUC) for both cynomolgus monkey and human given equivalent doses based on weight, 1 mg/kg, infused over a 2-h period, intravenously (average ± standard deviation, n = 3–6).

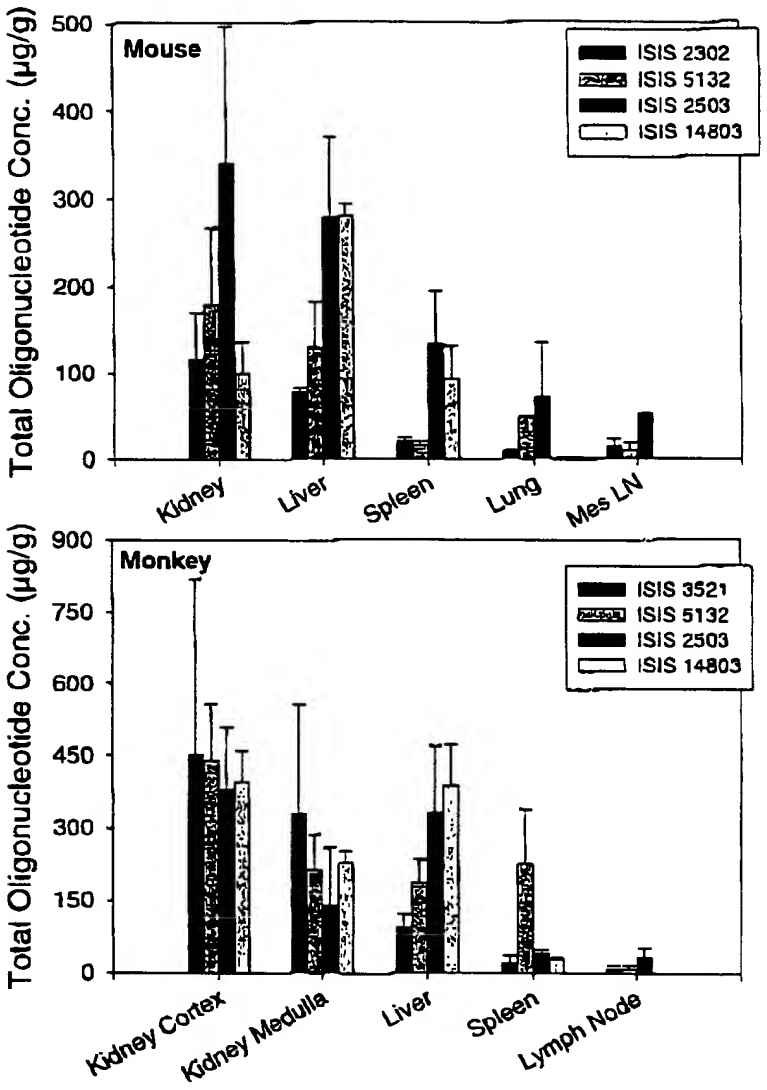

Figure 2 Phosphorothioate oligonucleotide concentrations in tissues from mouse and monkey. Mice were treated with ISIS 2302 for 2 weeks at 15 mg/kg every other day or 5132, 2503, or 14803 for 4 weeks at 20 mg/kg every other day and sacrificed 24 h after the last dose. Each bar represents the average of the concentration of total oligonucleotide in extracts from the respective tissues. Error bars are standard deviation of the mean. At least three animals are represented for each data point.

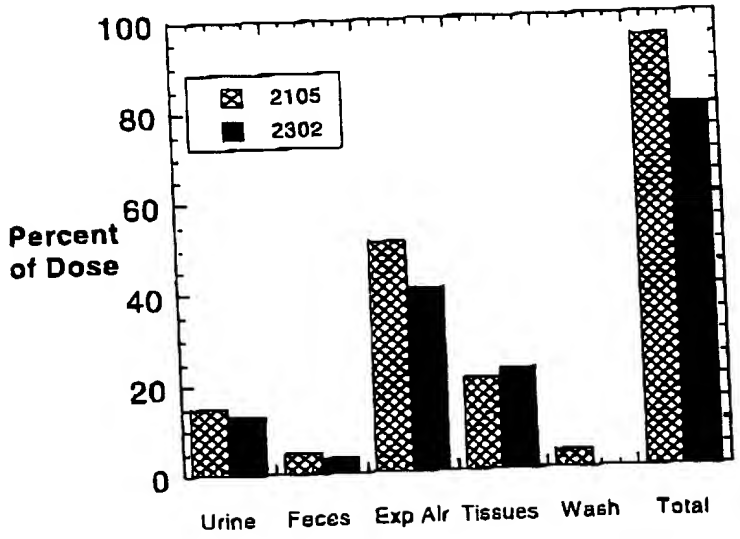

Figure 3 Percent of dose excreted in urine, feces, and expired air and remaining in tissue/carcass 10 days after single-dose administration of ^{14}C-labeled phosphorothioate oligodeoxynucleotides of two different sequences. ISIS 2105 is antisense to human papilloma virus mRNA and ISIS 2302 is antisense to human ICAM-1 mRNA. Both compounds are 20 mers. The radiolabel site is C-2 position of the thymidine nucleotides.

Thus, plasma pharmacokinetics and tissue distribution, clearance, and ultimately whole-body excretion of PS oligonucleotides have been shown to be sequence independent. This fundamental characteristic of these potentially useful therapeutic entities provides a clear path to rapid and safe development for multiple targets and varying diseases. It is likely that as we continue to explore the pharmacokinetics of this class of compound there will be sequences that exhibit some sequence-specific difference. Exonuclease metabolism rates examined in in vitro models, for example, differ in a sequence-specific manner (11). Additional research may provide evidence that such differences can also be seen in vivo.

III. PLASMA PHARMACOKINETICS

A. Intravenous Administration

After intravenous administration, phosphorothioate oligodeoxynucleotides are rapidly cleared from plasma. Plasma concentrations rapidly decrease following injection with distribution half-lives of 30–80 min (8,9,12). Radiolabel disposi-

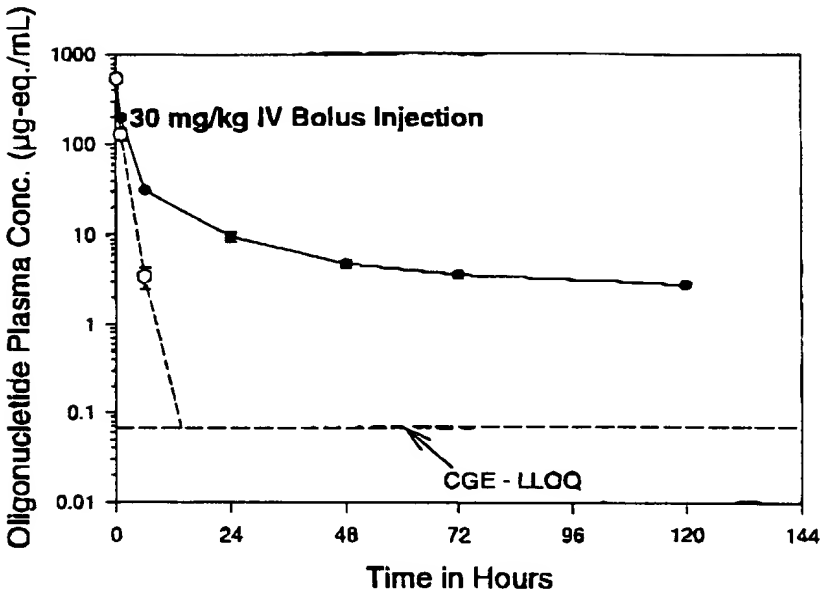

Figure 4 Radiolabel equivalent concentrations (filled circles) compared to parent oligonucleotide of ISIS 3521 (20-mer phosphorothioate oligodeoxynucleotide; assayed by CGE, open circles) concentrations following a bolus intravenous injection of ^{35}S-ISIS 3521 illustrates rapid distribution of parent oligonucleotide compared with prolonged circulation of presumed metabolites. Each time point represents the mean and standard deviation of three to six samples from separate animals.

tion is polyphasic with an additional much slower elimination phase with half-lives reported from 5 to as high as 75 h (Fig. 4), depending on the isotope used to label the oligonucleotide (13–18). The oligonucleotide-equivalent concentrations measured in plasma are several orders of magnitude higher than the intact drug concentrations measured by capillary gel electrophoresis (CGE) indicating that the radiolabel is no longer associated with the parent by later time points. Therefore, this much slower apparent terminal phase is likely a function of slow clearance of radiolabel from distributed tissue and as such appears to be associated with primarily low-molecular-weight metabolites of the original, parent PS oligonucleotide. For ^{14}C-labeled PS oligonucleotides, this slower elimination rate closely paralleled the clearance of oligonucleotide from tissue (17). The distribution portion of the plasma pharmacokinetic profile of intact oligonucleotide (parent) includes greater than 95% of the plasma AUC (internal data, unpublished). It can be deduced, therefore, that clearance of the parent oligonucleotide from

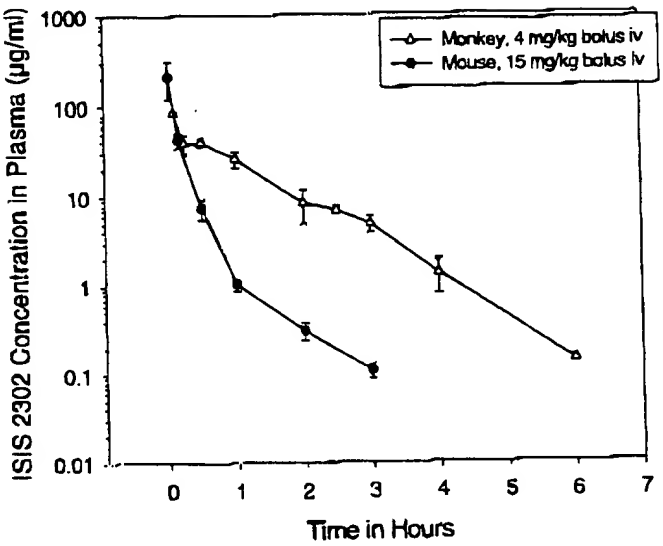

Figure 5 Plasma concentrations profiles for ISIS 2302 following bolus intravenous injections to cynomolgus monkeys and mice. Mice (three per time point) received a 15 mg/kg dose and monkeys (n = 3) received a 4 mg/kg dose.

plasma is a function of distribution to tissue and, to a lesser extent, exonuclease digestion/metabolism.

Plasma clearance rates are largely species independent in rat, rabbit, dog, and monkey and has been estimated to be between 1 and 3 mL/min/kg (8,9). Plasma clearance in mice has been shown to be somewhat faster than in other species (see Fig. 5), ranging from 8 to 14 mL/min/kg (19) (Yu et al., 2001, J Pharm Sci, in press). The more rapid plasma clearance in mice appears to be a function of more extensive exonuclease metabolism seen in mouse plasma (5) or more rapid tissue distribution due to more rapid circulation to tissues in mice. It is not clear which of these plays a dominant role, but it is clear that both of these mechanisms play a role in plasma clearance of PS oligonucleotides. Nevertheless, note that PS oligonucleotide pharmacokinetics scales well across species utilizing allometric correlations as a function of body weight (Fig. 6).

Intravenous infusion has been characterized in nonhuman primate toxicology studies to allow the study of higher doses while minimizing peak plasma concentrations (8,20). This intravenous dosing strategy has been employed in clinical studies for administration of PS oligonucleotides (21–24). After 2-h intravenous infusions in monkeys, maximum plasma concentrations are seen at or

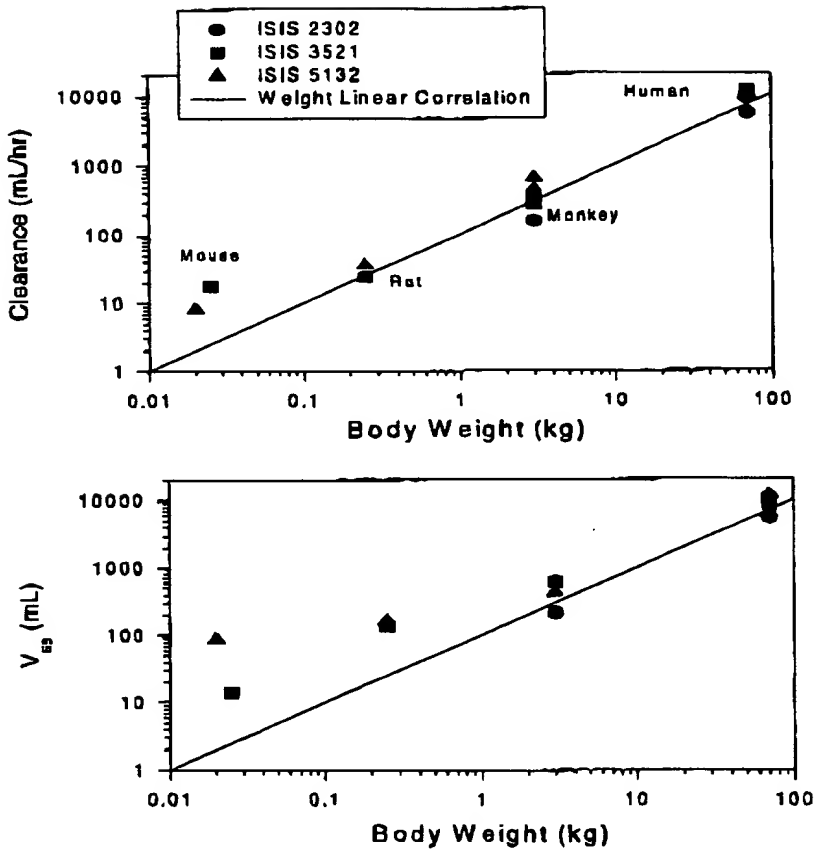

Figure 6 Allometric presentation of plasma pharmacokinetic data across species and different oligonucleotide sequences including ISIS 2302 for comparison. Each data point represents the mean of three to six animals or subjects. The doses used in the comparison were all <3 mg/kg. The slope of the line is 1 for both presentations, evidence that for all sequences the relationship is scalable directly by body weight.

near the end of the infusion period. The plasma concentrations at the end of the infusion are generally seen to be dose proportional. For example, in monkeys, plasma concentrations of 20 mer (parent oligonucleotide) were approximately 5, 15, and 50 μg/mL at the end of a 2-h infusion period for doses of 1, 3, and 10 mg/kg, respectively (8,9). After the infusion was stopped, PS oligonucleotide plasma concentrations decreased rapidly similar to that seen following intravenous bolus injection.

In mice, the plasma pharmacokinetics for doses ranging from 0.8 to 100 mg/kg appear to follow linear first-order pharmacokinetic principles with dose-proportional increases in maximum plasma concentrations and AUC (19). After repeated dose administration to mice every other day for 28 days, the plasma AUC and elimination half-lives increased at doses of 20 and 100 mg/kg, suggesting a change in clearance. After repeated administration at lower doses in mice and monkeys for up to 6 months, no change in plasma clearance was seen (internal data, unpublished). However, the area under the plasma concentration-time curves (AUC) increased greater than proportional for single doses administered to rats and monkeys at doses ranging from 1 to 30 mg/kg (Table 2). Collectively, these data suggest saturation of some disposition pathway at the higher doses that appears to occur only after repeated dosing in mice but is observed acutely in other animal species including humans (22). These nonlinear kinetics are characterized by slower apparent distribution half-lives at higher doses and a slowing in overall plasma clearance at higher doses. As will be described in metabolism sections in greater detail, it is now evident that the extent and rate of metabolism in vivo of PS oligonucleotides in plasma are not affected by increased dose (19) (Yu et al., 2001, J Pharm Sci, in press). However, distribution to organs that take up a large fraction of the oligonucleotide dose (liver and kidney) appears to saturate as dose increases (25–27). Taken together, these data suggest that the nonlinear behavior observed in plasma clearance may be related to saturation of distribution to major organs of distribution.

Longer-term continuous infusion may have the advantage of providing prolonged exposure of the oligonucleotides to organs that do not readily take up oligonucleotide. Some initial evidence of this was observed in early continuous infusion studies reported by Iversen et al. (28). A direct comparison of rapid infusion and continuous infusion of ISIS 3521 in rats and monkeys has confirmed that higher tissue concentrations can be achieved (Geary et al., in preparation). These increases in tissue concentration are achieved in both major organs of distribution that exhibit saturable distribution and other tissues and organs that take up much smaller fractions of the oligonucleotide dose (Fig. 7). Upon continuous infusion, plasma concentrations of oligonucleotide achieve steady state by 8 h in rats and monkeys, consistent with rapid clearance from plasma. After 5 days of continuous infusion, the concentrations remain constant in plasma and then fall rapidly after the infusion is stopped. There does not appear to be any change in clearance even after 5 days of continuous infusion in rats (Fig. 8). These data taken together suggest that saturation of distribution may be an acute equilibrium-binding phenomenon on the surface of cells and tissues rather than true saturation of uptake into the organ or tissue. As will be discussed in more detail in the following sections, uptake in cells associated with tissues of distribution is believed to occur in sequential steps that require initial binding to tissue proteins followed by as yet poorly understood active or passive internalization into cells.

Table 2 Summary of Dose-Dependent Plasma Pharmacokinetics Observed for Two 20-mer PS Oligonucleotides in Rat and Monkey

Parameter	Rat ISIS 2302 (ICAM-1)		Rat ISIS 3521 (PKC-α)		Monkey ISIS 2302 (ICAM-1)		Monkey ISIS 3521 (PKC-α)		
dose (mg/kg)	4	20	3	30	1	4	1	3	10
C_{max} (μg/mL)	52	315	54	591	4.6	30	4.3	20.8	52.3
AUC (μg/h/mL)	39.2	290	45	676	9.67	77.5	8.10	37.2	110
$t_{1/2\beta(obs)}$ (min)	28	56	61	90	28	57	80	70	155
Cl_p (mL/min/kg)	1.70	1.15	1.10	0.74	1.72	0.86	2.06	1.34	1.51

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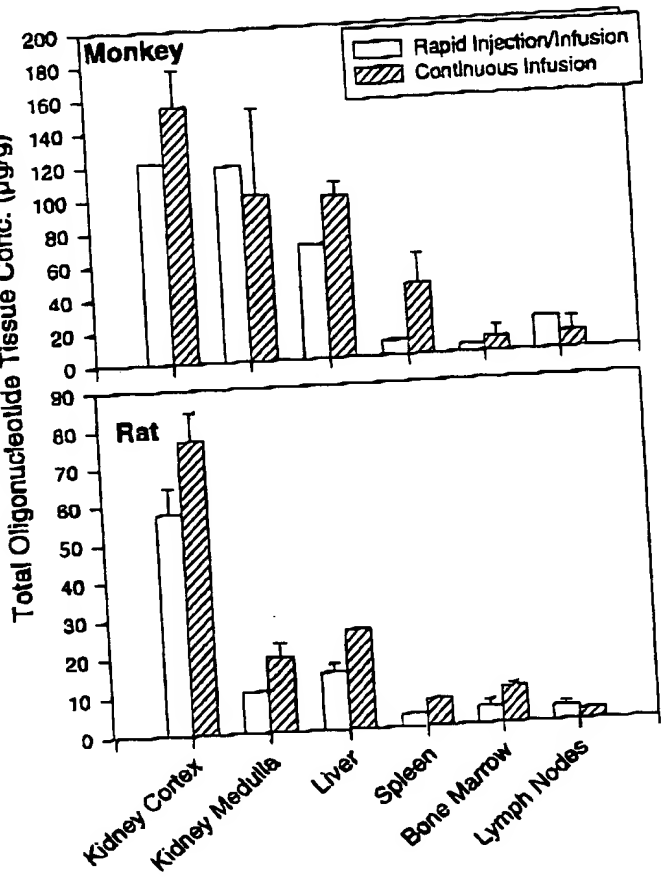

Figure 7 Increased distribution of oligonucleotide to tissues following continuous intravenous infusion of ISIS 3521 in monkeys and rats when compared with shorter-duration infusions (2-h infusion in the monkey) or bolus injection (rat). The tissues were collected at sacrifice 24 h after the dose was administered. Each bar represents the average of three to six animals [except for the 2-h infusion arm (open bar) of the monkey study. $n = 2$]. Error bar is the standard deviation. Dose: 3 mg/kg.

B. Absorption

Although oligonucleotides are hydrophilic, relatively large, and have multiple charges at physiological pH, antisense phosphorothioate oligodeoxynucleotides are remarkably well absorbed from intradermal and subcutaneous sites of injection (10,18,26,29,30). Low and dose-dependent systemic absorption is seen following intratracheal administration as well as via pulmonary delivery using aero-

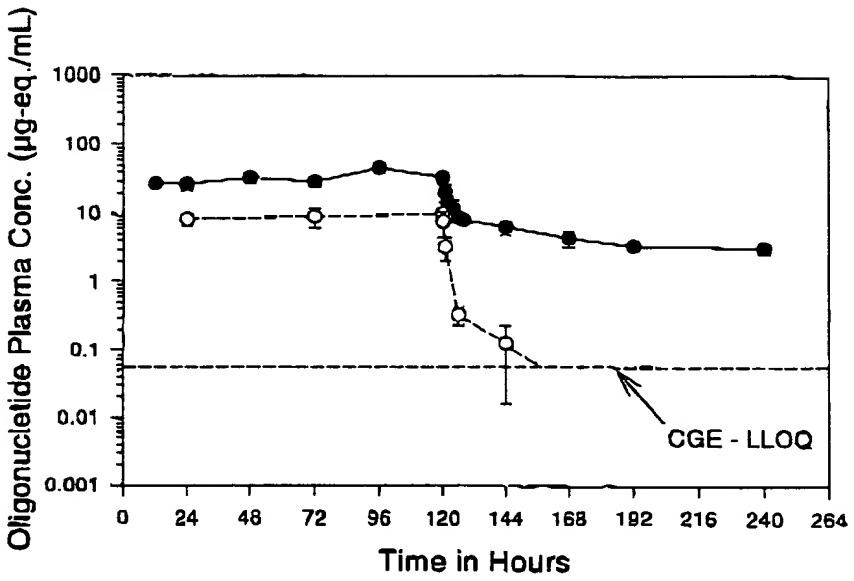

Figure 8 Plasma concentrations of radiolable equivalent oligonucleotide (filled circles) compared with intact ISIS 3521 (20-mer PS oligonucleotide, assayed by CGE, open circles) during a 5-day continuous infusion study in rats. Steady-state concentrations of parent compound as well as total radiolabel were achieved by 24 h consistent with the rapid disposition kinetics. Each time point represents the average and standard deviation of three to six samples from separate animals.

solized liquid containing oligonucleotide (30) (Templin et al., 2000, Antisense Nucleic Acid Drug Dev, in press). Nevertheless, oral absorption of this class of compounds is poor likely due to the extreme conditions encountered in the gastrointestinal tract resulting in poor stability coupled with poor permeability across the gastrointestinal epithelium (10,31,32) (Geary et al., 2001, in press).

1. Parenteral Administration

Treatment of systemic conditions by direct intravenous injection of antisense PS oligonucleotides bypasses absorption barriers for delivery of these compounds. However, peak plasma concentrations generated by rapid injection directly into venous blood is prohibited by hemodynamic effects that occur at high oligonucleotide plasma concentrations. Therefore, slow intravenous infusion (either 2-h or

continuous infusion over days) has been adopted for direct systemic delivery. These regimens have the disadvantage of being inconvenient for both the patients and the health-care professionals caring for the patients.

Intradermal injection of ^{14}C-labeled ISIS 2105 (human papilloma virus antisense) was explored as a potential route for local treatment of genital warts. However, ISIS 2105 was rapidly absorbed from the injection site (18) in rats with approximately 65% of the dose absorbed within the first hour. Peak plasma concentrations were observed within 30 min. This was later confirmed in clinical trials (29). While this represented a failed approach to localization of the oligonucleotide, it provided a clue that delivery into well-perfused regions in the body could result in nearly complete absorption of these compounds.

Subcutaneous administration of PS oligonucleotides likewise results in nearly complete absorption of these compounds over time (26,30). Subcutaneous injection also slows the input of oligonucleotide as compared to direct intravenous injection and thus results in lower maximum plasma concentrations (Fig. 9). This characteristic of subcutaneous administration is attractive for clinical purposes because it allows for rapid injection along with the convenience of self-administration without inducing the high plasma concentrations associated with established threshold for hemodynamic changes seen in monkeys (33,34). Although evidence of complement activation in the clinic has not materialized to date, the added convenience of self-administration should not be minimized. Furthermore, subcutaneous administration of PS oligonucleotides has been implemented in long-term safety studies 1–6 months in duration when repeated intravenous administration was not practical (internal data, unpublished).

2. Nonparenteral Administration

Pulmonary, oral, and rectal routes of administration for PS oligonucleotides have been explored. Intratracheal administration in rats resulted in significant, albeit dose-dependent bioavailability of ISIS 3521 (CGP 64128A) (30,35). The dose-dependent absorption may be the result of local irritation or inflammation caused by high localized concentrations of PS oligonucleotides. However, experiments with permeability markers appear to suggest that functional integrity of the tracheal or lung epithelia was not compromised. Thus, the increased absorption at higher doses may be related to a saturable absorption-limiting local tissue binding in the lung. Direct pulmonary delivery to mice using aerosolized "naked" PS oligonucleotide, ISIS 2105, administered at doses of 1.2–12 mg/kg, also resulted in dose-dependent systemic absorption. In this experiment, however, mild inflammatory response was observed at the 12-mg/kg dose. At doses at or below 3 mg/kg, toxicity of the lung was minimal to absent and resulted in excellent local exposure of most cell types in the lung (Templin et al., 2000, Antisense

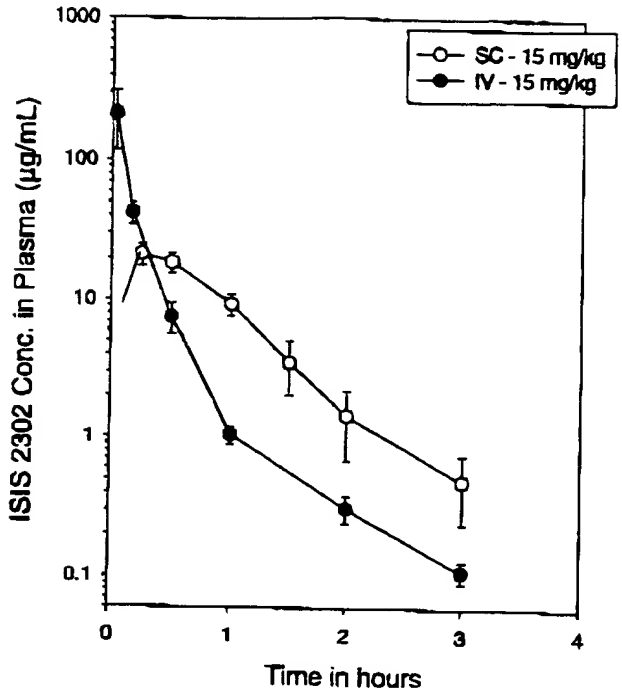

Figure 9 Plasma concentration profiles of intact ISIS 2302 (20 mer) as a function of time following single subcutaneous or intravenous bolus administration of 15 mg/kg in mice. Each time point represents the average of three animals. The error bars are standard deviation.

Nucleic Acid Drug Dev., in press). Other reports have shown impressive data supporting the utility of direct pulmonary delivery of antisense PS oligonucleotides for treatment of inflammatory diseases such as asthma (36,37).

Oral administration represents the most desirable route of administration for the convenience of treatment in the clinic, particularly for chronic diseases. However, the oral bioavailability of PS oligonucleotides is poor, with less than 1% of a radiolabeled dose absorbed (10,30). The permeability of PS oligonucleotides has been shown to be low using both in vitro methods (31) and an in situ rat intestinal method (32).

Improvement in permeability on the order of two- to fourfold was achieved by chemical modifications that will be common for the next generation of antisense oligonucleotides (38). We have also characterized the stability of PS oligonucleotides in the gastrointestinal tract and it is clear that they are susceptible to

rapid nuclease digestion. In the rat intestine, the half-life of oligonucleotide is approximately 1 h with no measurable intact oligonucleotide in the intestine by 8 h after administration (Geary et al., 2001, J PET, in press). These data suggest a substantial preabsorptive barrier to successful bioavailability of PS oligodeoxynucleotides. Once again, the second-generation modified oligonucleotide chemistries that afford much improved stability to nuclease digestion are likely to provide a more reasonable opportunity for success by this route of administration.

C. Plasma Protein Binding

Phosphorothioate oligodeoxynucleotides readily bind to plasma proteins. At clinically relevant doses, more than 96% of PS ODN in plasma is bound to plasma proteins in mice, rats, monkeys, and humans (Fig. 10). For all species tested, there is little change in binding over the concentration range from approximately 1 μg/mL to 68 μg/mL (Watanabe et al., 2000, manuscript in preparation). Albumin and α_7-macroglobulin are the major protein species that bind ISIS 2302 with relatively high capacity (39). Affinity of binding has been characterized for these two proteins using different analysis methods. Electrospray–mass spectrometry (ES-MS) provided estimates for dissociation constants of 3.1 μM (K_{D1}) and 11.9

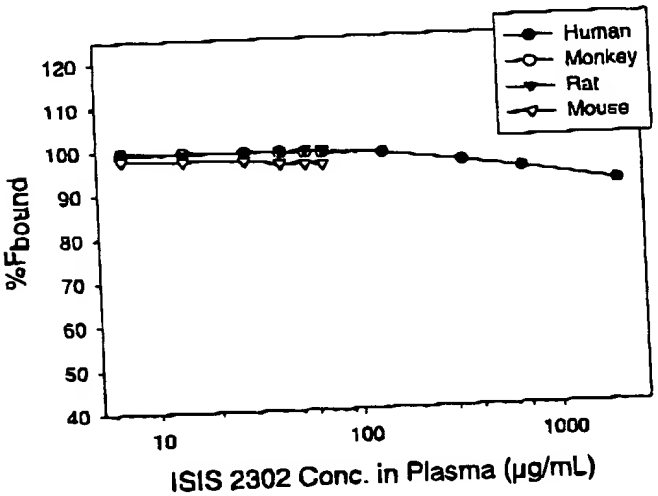

Figure 10 Comparison across species of fraction of ISIS 2302 in plasma that is in the bound form. Protein-binding experiments were conducted in fresh whole plasma. Each analysis involved five replicates and the error bars represent standard deviation of the mean.

μM (K_{D2}) for albumin (40). Binding to plasma proteins has been shown to be salt- and pH-dependent, suggesting that the binding is likely a nonspecific electrostatic interaction. The binding affinity of PS ODN to α_2-macroglobulin was found to be greater than albumin. Using surface plasma resonance (SPR), the dissociation constants were determined to be 4 nM (K_{D1}) and 72 nM (K_{D2}) for α_2-macroglobulin (internal data, unpublished) for another 20-mer phosphorothioate oligodeoxynucleotide.

In contrast, binding a 20-mer PS ODN, ISIS 2302, to α_1-acid glycoprotein (AAG), another high-capacity plasma protein, was negligible (internal data, unpublished). The levels of AAG in plasma of Crohn's disease patients is known to be increased (41) and these altered levels can affect the kinetics of drugs bound to AAG. Such is the case with the β blocker propanolol. The apparent lack of oligonucleotide binding to AAG makes it unlikely that AAG alterations in Crohn's patients will impact the pharmacokinetics of antisense oligonucleotides administered to this patient population.

Phosphorothioate oligonucleotides have been shown to bind to other less abundant plasma proteins, sometimes with high affinity. For example, SPR analysis demonstrated that PS ODN bind to thrombin-binding sites with relatively high affinities of 30 nM (K_{D1}) and 230 nM (K_{D2}) (internal data, unpublished). Factor H (33) and components of the intrinsic tenase complex (42) have also been shown to bind with PS ODN. The binding of PS oligonucleotides to these proteins in serum at high concentrations of oligodeoxynucleotides appears to disrupt normal enzymatic function and likely explains acute hematological effects observed in monkeys (33).

Protein binding may explain many other pharmacokinetic properties of ISIS 2302. The high degree of protein binding in circulation prevents any significant urinary excretion. Therefore, there is little glomerular filtration of the protein-bound oligonucleotide and the excretion of intact compound in urine is a minor pathway for its clearance from plasma (19). In mouse toxicology studies with doses ranging from 0.8 to 100 mg/kg, urine excretion of intact oligonucleotide was seen to be dose dependent as increased excretion was observed with increased dose. Thus, urine excretion occurred as concentrations of oligonucleotide exceeded the capacity of the plasma binding. Only after bolus intravenous injection of high doses (20 mg/kg) of ISIS 5132 in mice are plasma concentrations high enough to increase unbound fraction of oligonucleotide resulting in urinary excretion of intact oligonucleotide. However, saturation of plasma protein binding occurs only at plasma concentrations many fold higher than those achieved at therapeutic doses in the clinic (Watanabe et al., manuscript in preparation). The negligible renal excretion of oligonucleotide provides greater opportunity for PS oligonucleotide to distribute to peripheral tissues, the site of action.

Protein binding of PS oligonucleotide is reversible and exists as an equilibrium between high-affinity and low-affinity proteins. Some protein binding is

associated with plasma while other binding occurs in tissue as well. Tissue distribution is attributed to protein binding and the binding of higher-affinity proteins in organs and on the cell surface. Once bound, oligonucleotide is internalized in the cell. Thus, both whole-body distribution and cellular uptake of PS oligonucleotide and other oligonucleotides are highly dependent on protein binding (S. Crooke et al., JPET paper, in press). It is tempting to speculate that the observed saturable distribution characteristics of PS ODN may be associated with saturation of binding to proteins in specific tissues.

Note that extremely high concentrations of aspirin (2 mg/mL in plasma of treated rats) have been reported to displace another 25-mer PS oligonucleotide from whole-rat plasma proteins, increasing free fraction from approximately 6% to approximately 11% (43). The dose of aspirin required to achieve displacement of the PS oligonucleotide in vivo was 50 mg/kg. Increasing the free fraction of PS oligonucleotides resulted in decreased exposure of oligonucleotide in plasma and in tissue with concomitant increases in urinary and fecal excretion. Thus, displacement of oligonucleotide from plasma results in increased excretion and an overall decrease in exposure in plasma and tissue. However, the concentrations of aspirin required to accomplish this effect were many times higher than are clinically relevant. The mechanism for displacement was not examined. Nevertheless, these data point to the need for further understanding of the interaction of PS oligonucleotide binding with other drugs that bind to serum albumin or other relevant plasma proteins.

IV. TISSUE DISTRIBUTION AND CLEARANCE

Rapid clearance of PS oligonucleotides from plasma occurs with a concomitant appearance in tissues in mice, rats, and monkeys (13,16,19,26,44). The highest concentrations of PS oligonucleotides in all species studied were found in kidney, liver, spleen, and lymph nodes (Fig. 11), but PS oligonucleotide can be measured in almost every tissue, except brain, shortly after intravenous administration. Cell-specific distribution within kidney and liver has been extensively studied (45–52). Once again, the similarity between the distribution observed for PS oligonucleotides in various species provides evidence that the pharmacokinetics are largely driven by their chemical class and not by their specific sequence (9). The consistency of tissue distribution of PS oligonucleotide between species is thought to be the primary reason for the similarity in plasma kinetics between species.

Cellular distribution of PS oligonucleotides within various organs has been described using autoradiography (26,52–54), immunohistological methods (54–56), and suborgan as well as subcellular physical separations coupled with capillary gel electrophoresis (51).

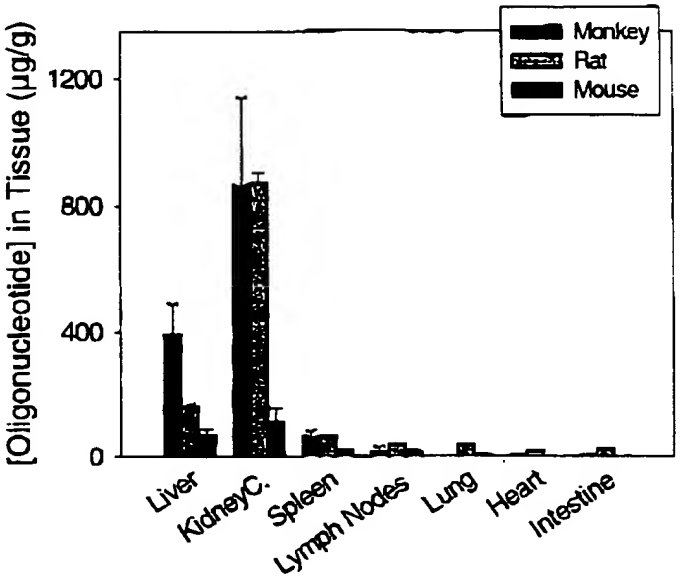

Figure 11 Comparison of the tissue concentration profiles for total oligonucleotide associated with ISIS 2302 across species. The monkey tissues were collected 48 h after the last dose following 28 days of dosing of alternate-day dosing of 4 mg/kg. The rat tissues were collected 48 h after the last of four doses administered on an alternate-day schedule for 1 week at a dose of 20 mg/kg. The mouse tissues were collected 24 h after the last of seven alternate-day doses of 15 mg/kg given over a 2-week period. Each bar represents at least three animals. Error bars are standard deviation.

Once PS oligonucleotides are distributed into tissues, their clearance is relatively slow (17,19,20). Initial distribution into tissues is associated with binding of the oligonucleotide to extracellular matrix, interstitium, or loosely bound to the cell membrane. However, by 4 h after intravenous administration in rats approximately half of the oligonucleotide measured in the liver was located intracellularly (51). By 24 h after injection very little oligonucleotide remains bound to extracellular components. Thus, it is likely that whole-organ pharmacokinetic evaluation after 24 h will represent intracellular exposure and whole-organ clearance will parallel cellular clearance. Tissue half-lives were comparable across species with the longest clearance half-lives seen in monkeys (Table 3). The relative rates of tissue elimination of ISIS 2302 in rats and mice and ISIS 3521 in monkeys are consistent with that reported for other PS ODN (17–19).

Thus, while PS oligonucleotides are cleared quickly from blood, their residence time in tissues in all species examined is relatively long. The relatively

Pharmacokinetic Properties in Animals

Table 3 Comparison of Tissue Elimination Half-Lives (in Days) for Intact Oligonucleotide in Rodents and Monkeys

	Intact PS oligonucleotide half-life (in days)		
Tissue	Mouse[a]	Rat[a]	Monkey[b]
Kidney	2.8	4	5
Liver	1.8	2.6	2.8
Spleen	0.7	0.9	3.8
Lymph nodes	0.6	1.7	0.9

[a] ISIS 2302.
[b] ISIS 3521.

slow clearance from tissues is favorable for the intended pharmacological application, and alternate-day administration appears to maintain oligonucleotide concentrations sufficient to produce pharmacological activity. With half-lives estimated between 1 and 5 days, accumulation factors should range from 1.5- to approximately threefold depending on the tissue or organ, if the pharmacokinetics follows first-order, linear pharmacokinetic principles. In fact, the accumulation observed in mice and rat appears to be predicted based upon these principles.

A simulation exercise was undertaken based on the assumption that the tissue half-lives calculated for one sequence are relevant to another. The tissue concentrations for ISIS 2302 were simulated using tissue half-life data obtained for ISIS 3521 in monkey (25). Half-life in tissues determined on the basis of single-dose kinetics of ISIS 3521 accurately predicted the steady-state concentration for ISIS 2302 at the end of 1- or 6-month toxicity studies, and also predicted peak and trough concentrations produced by an intermittent dose schedule (Fig. 12). Slow clearance from tissues is also consistent with the apparent accumulation in target organs following every-other-day administration. The fact that accumulation in tissues occurs with repeated administration suggests that the saturation observed in liver as dose increased was a saturation of tissue distribution kinetics specifically, and not a saturation of the capacity of the organ to store oligonucleotide.

Although accumulation of ISIS 2302 and total oligonucleotide occurred with every-other-day administration, steady-state concentrations were achieved within 4 weeks of treatment (internal reports, unpublished). In subchronic and chronic treatment of monkeys, tissue concentrations of ISIS 2302 were the same at 28 days, 3 months, and 6 months of treatment, suggesting steady state was achieved within 4 weeks of treatment (internal data, unpublished). Furthermore,

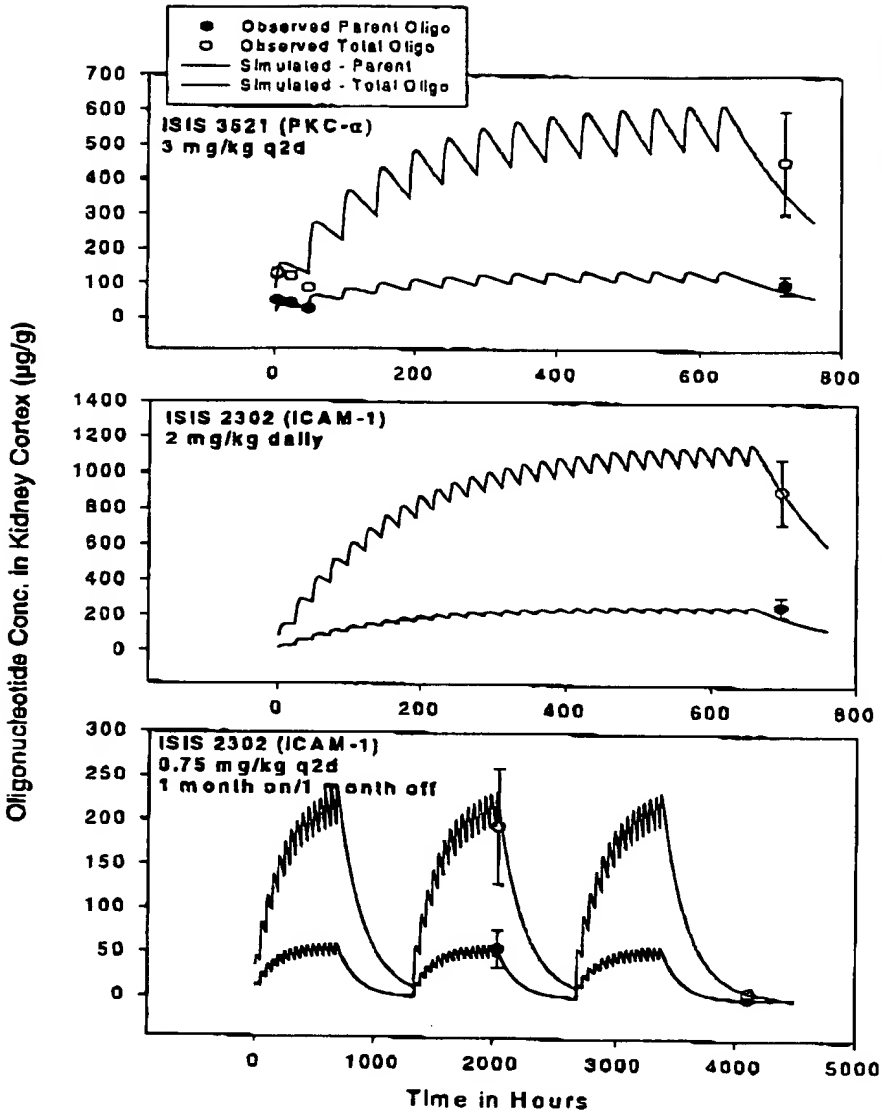

Figure 12 Simulated oligonucleotide concentrations compared with measured concentrations in monkey kidney cortex following repeated intravenous dosing by either alternate-day or daily dosing regimens. (Top) Data for ISIS 3521—28 days of dosing every other day; (middle) data for ISIS 2302—28 days of dosing daily; and (bottom) data for ISIS 2302 doses on alternate days for 1 month on and then 1 month off, repeated three times. Both total and parent oligonucleotide are represented in this simulation.

it is clear that steady-state concentrations were predicted on the basis of single-dose kinetics of a related 20-mer PS ODN.

Data from repeat-dose pharmacokinetic studies indicate that tissue concentrations of PS oligonucleotides can be maintained with every-other-day, or, by inference, 3-times-per-week treatment regimens. Thus, these dose regimens are appropriate for toxicity evaluation or therapeutic activity, because they maintain exposure to PS oligonucleotides in target organs for the duration of treatment and for some period of time thereafter. Complete clearance from tissues is predicted to occur by 2–4 weeks after cessation of dosing.

Although tissue exposure was dose-dependent, tissue concentrations in liver, and to some degree in kidney, increased less than proportional to dose (Fig. 13). For example, liver concentrations increased only fivefold as the dose increased from 2 to 20 mg/kg (22 ± 13 μg/g and 122 ± 23 μg/g, respectively). Nonlinear tissue distribution was observed for both the parent oligonucleotide and total measurable oligonucleotide (including chain-shortened metabolites). Since liver accounts for the greatest percentage of dose distributed to tissue, saturation of the oligonucleotide distribution correlates with greater than dose-proportional increases in plasma C_{max} and AUC values together with a decrease in plasma clearance described earlier.

V. METABOLISM AND EXCRETION

Metabolites of PS ODN are evident in both plasma and tissues almost immediately after dosing. Metabolism occurs predominantly through exonuclease-mediated cleavage of nucleotide residues from the parent oligonucleotide (3,5,6). There is no evidence that hepatic microsomal enzymes play a significant role in the elimination of this class of compounds (11). Exonucleases cleave single nucleotides from either the 3′ or 5′ end of the molecule, liberating mononucleotides and oligonucleotide metabolites that are shortened by one nucleotide. The first oligonucleotide metabolite is 19 nucleotides in length, and is referred to as a 19-mer or the N-1 metabolite. This metabolite continues to be shortened until it is completely degraded or the "shortmers" are excreted in the urine. Evidence for this progressive metabolism is found in electropherograms showing the profile of metabolites over time (Fig. 14). The metabolism is progressive such that an N-1 metabolite is the primary oligonucleotide metabolite followed by N-2, N-3, etc., as more nucleotides are sequentially removed.

The metabolic fate of PS ODN follows many, if not all, of the same pathways as endogenous nucleotides, nucleosides, and bases. Studies with PS oligonucleotides have exploited different radiolabeling schemes and a number of bioanalytical techniques to obtain a thorough understanding of the metabolic fate of the nucleotides and bases, as well as sulfur on the thioate linkage, the xenobiotic

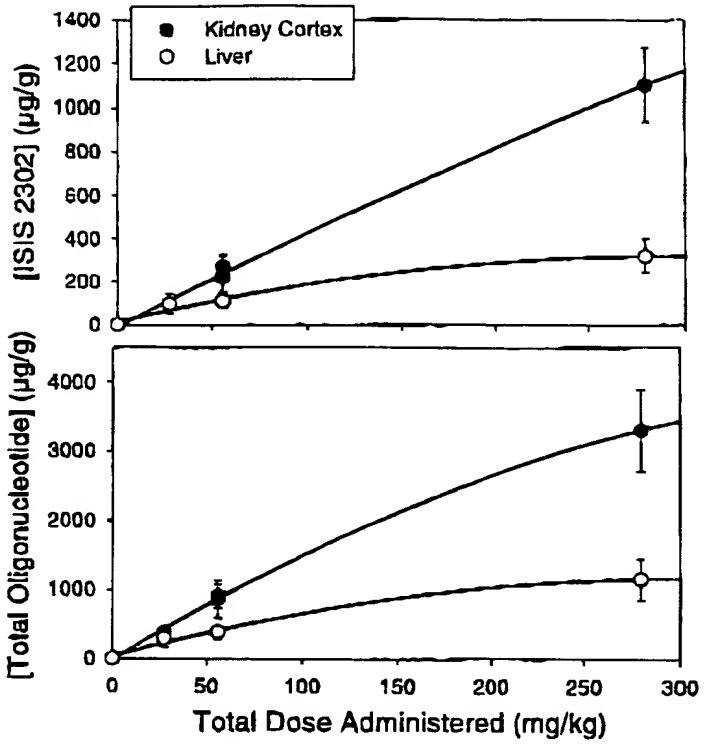

Figure 13 Distribution of oligonucleotide to liver and kidney as a function of total administered dose. ISIS 2302 and total oligonucleotide concentrations in liver and kidney from cynomolgus monkey are plotted against total dose administered. The doses were given as follows: 2 mg/kg given every other day for 28 days (total dose of 28 mg/kg), 4 mg/kg given every other day for 28 days (total dose of 56 mg/kg), and 20 mg/kg given every other day for the same period (total dose of 280 mg/kg). Approximately a 10-fold increase in dose resulted in less than a fourfold increase in ISIS 2302 concentrations in liver. Similar decrease in the amount of total measurable oligonucleotide taken up by liver tissue was seen over this dose range.

component of PS ODN. Mass balance studies using ISIS 2302 radiolabeled at the C-2 position of the thymines provided significant information on the metabolism of the oligonucleotide and the specific fate of the pyrimidine bases. In these studies, all excreta and expired air were collected from rats treated with ^{14}C-labeled ISIS 2105 (17,18). Liberation of $^{14}CO_2$ was the primary route of clearance of pyrimidine-derived radiolabel. Approximately 50% of the radiolabel is cleared over the course of 10 days as $^{14}CO_2$. Two other 20-mer PS ODN of different

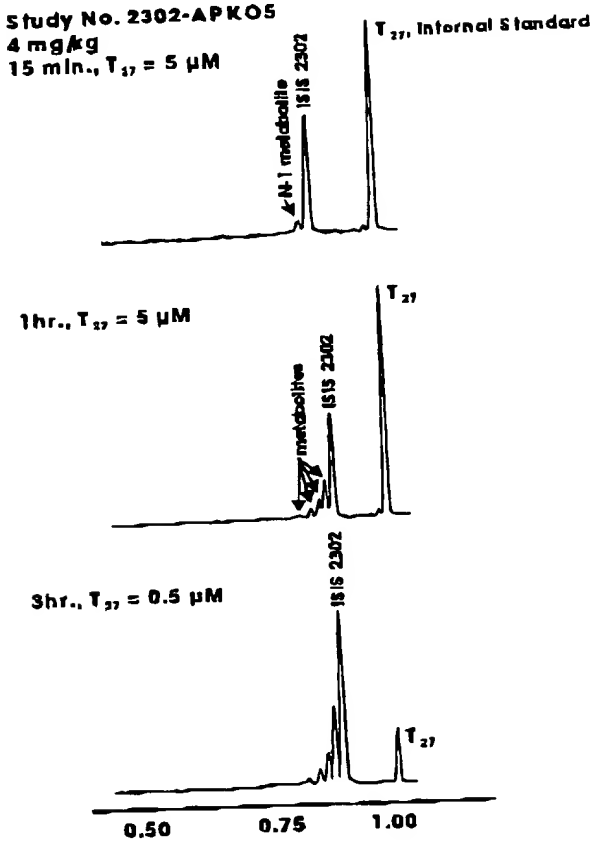

Figure 14 Capillary gel electropherograms of plasma taken from monkeys following a 4 mg/kg dose of ISIS 2302. T_{27} is a 27-mer deoxythymidine added as an internal standard at the concentration indicated.

sequence (ISIS 2922 and ISIS 2302) but equivalent label type and position have exhibited similar mass balance profiles of metabolic fate (internal data, not published). Taken together, these data suggest that the pattern of metabolism is common for the class.

A. Nuclease Metabolism

The successive removal of bases from the 3′ end of PS ODN is the major pathway for metabolic degradation in plasma (4,5), while both 5′ and 3′ exonuclease exci-

sion may occur in tissues (2,3,6). LC/MS and end-labeling techniques have been applied to allow differentiation and identification of the shortened metabolites.

Thus, exonuclease-mediated metabolism produces a metabolic pattern of progressively shorter metabolites each shortened by a single nucleotide. Metabolism by endonucleases would be expected to produce a metabolite pattern characterized by an irregular distribution of shortened metabolites and an enrichment of metabolites cleaved at specific intervals; for example, a 10-mer metabolite might be observed in the absence of the 11- or 12-mer. Phosphorothioate oligonucleotide metabolites consistent with endonuclease activity have not been observed in studies with PS ODN (5,11,19).

As PS ODNs are metabolized to shorter oligonucleotides after intravenous administration, the percent of oligonucleotide in plasma that is represented by full length (20 mer) drops from approximately 91% immediately following intravenous administration, to 65% by 10 min after injection in mice (19). Subsequent to this initial rapid shortening, however, the percentage of full-length ISIS 2302 slowly decreases over the next 3–4 h (Fig. 15). This apparent change in metabolic rate over time is observed in every species studied including human (21). The biphasic nature of the metabolism profile in plasma is poorly understood, but is thought to be related to the stereochemistry of a PS ODN.

The sulfurization process produces a random distribution of R and S chiral

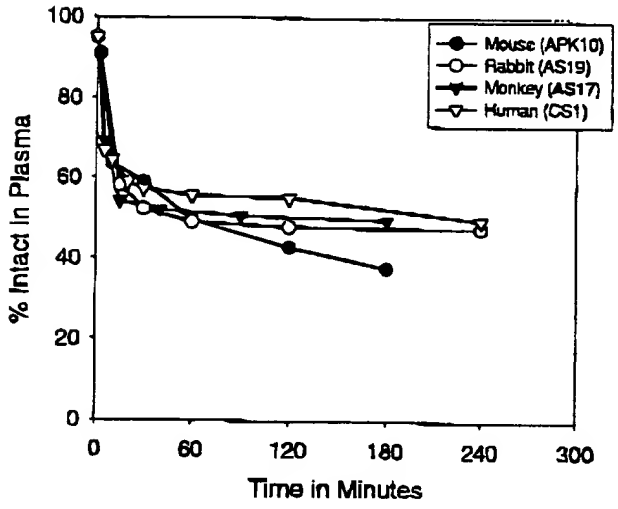

Figure 15 Rate and extent of metabolism seen in plasma and compared across species. Percent intact is the percent of total measurable oligonucleotide represented by parent ISIS 2302 (20 mer). Each point represents the average of three to six individuals.

centers at each phosphorothioate linkage. These are referred to as Rp and Sp centers. Therefore, phosphorothioate oligodeoxynucleotides are racemic mixtures of Rp- and Sp-phosphorothioate linkages, with approximately 2^{19} isomers in the mixture. The rate of exonuclease cleavage for the Rp stereoisomer is much more rapid than for the Sp isomer (57–59). The rate of metabolism of any single molecule of ISIS 2302 is expected to be relatively rapid until the exonuclease reaches an Sp linkage at which point it would slow. In plasma, where metabolism is almost exclusively from the 3′ terminus, this phenomenon would tend to favor ISIS 2302 metabolites that have a 3′ terminal Sp linkage. If approximately 50% of the linkages are Sp, then it would follow that metabolism would slow as the population of oligonucleotides became more and more enriched with oligomers that have the more slowly metabolized Sp linkage. Because approximately 50% of the oligonucleotide administered will have an Sp linkage at the 3′-most phosphorothioate linkage, metabolic slowing would be reached when approximately 50% of the parent compound remains.

In the absence of techniques to differentiate between stereoisomers it has not been possible to demonstrate that this enrichment actually occurs. However, kinetic modeling was performed to determine whether metabolic preferences might result in a pharmacokinetic profile like that observed in vivo. The pharmacokinetic model was built assuming a probability of 0.5 at each linkage for rapid (3-min Rp) or resistant (3-h Sp) cleavage (19). The kinetic pattern produced by this model is very similar to that observed in vivo, suggesting that stereochemical differences in metabolic rate could, at least theoretically, produce the metabolic slowing that is observed in plasma in all species. The biphasic profile of nuclease metabolism observed in the plasma of all species may, therefore, be explained by a differential rate of metabolism for the Rp and Sp diastereoisomers present at each phosphorothioate linkage of ISIS 2302. Note that this was observed in all species exposed to the drug, such that both toxicity studies and clinical trials evaluated the drug under conditions where there was the potential for this stereochemical enrichment.

Intact ISIS 2302 is always the most abundant oligonucleotide species observed in plasma or tissue following intravenous or subcutaneous administration in mouse or monkey. After repeated administration of PS oligonucleotide at doses ranging from 0.8 to 100 mg/kg in mice (19) and 1 to 10 mg/kg in monkeys (R. Yu et al., 2001, J Pharm Sci, in press), the metabolite pattern observed in plasma is unchanged suggesting that metabolism in the circulation is neither inhibited nor induced. Indeed the pharmacokinetic profile of oligonucleotide metabolites shortened by one or more nucleotides was similar to that of the parent oligonucleotide, ISIS 2302 (Fig. 16). Also, the pattern of metabolites in tissue was not altered after repeated every-other-day intravenous administration.

Intact PS ODN represented approximately 20–40% of the total oligodeoxynucleotide measured at 24 h or 72 h after dose administration in most mouse

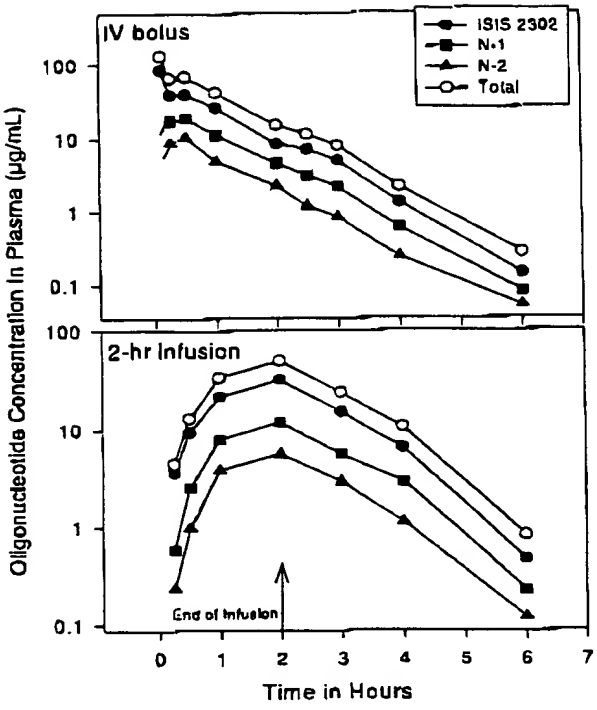

Figure 16 Plasma profiles for parent drug (ISIS 2302) and its shortened oligonucleotide metabolites in monkeys following bolus and 2-h intravenous infusion at a dose of 4 mg/kg (Study No. 2302-APK05). Each data point represents the average of three monkeys.

tissue (19,26). The remainder of the oligonucleotide that could be effectively measured was in the form of 19 to 16 mers. While oligonucleotides shorter than these have been observed, the levels detected were low and their contribution to the oligonucleotide burden was not great. The metabolites of PS oligonucleotides in tissue tended to diminish in parallel with one another such that the fraction detected intact diminished only slightly with time. In monkeys after intravenous administration of 4 mg/kg, intact ISIS 2302 represented approximately 45–60% of the total oligodeoxynucleotide measured in liver and kidney at 4 h and 35–45% after 48 h (internal data, not published).

Metabolites have been identified in tissues with elution profiles that could not be the result of single nucleotide excision. These metabolites, found exclusively in tissues, migrated more slowly than the parent oligonucleotide suggesting greater mass or lower charge-to-mass ratio. Mass spectral analysis of these metabolites demonstrated fragmentation patterns that were consistent with the addition

of one or more nucleotide bases to the parent oligodeoxynucleotide (60). Typically these slow-migrating metabolites comprised less than 10% of the total oligodeoxynucleotide measured at 24–48 h after administration. It is unknown whether these metabolites are pharmacologically active although at least one-fourth of the elongation forms should be a perfect match to the mRNA sequence, suggesting that there should be some pharmacological activity of these forms. These larger metabolic products clear from tissues along with ISIS 2302 and the other shortened oligonucleotide metabolites in mice at similar rates (internal data, unpublished).

B. Excretion

For each chain-shortening event one mononucleotide is release and some fraction of the oligonucleotide should be excreted as a low-molecular-weight metabolite in urine. In studies with radiolabeled PS oligonucleotide, there is evidence for low-molecular-weight metabolites in plasma, urine, and tissues that are poorly retained on anion-exchange HPLC and elute near the void volume (13). In studies utilizing ^{14}C-labeled ISIS 2302, more than 90% of the radiolabel recovered in urine is associated with these poorly retained metabolites (internal data, unpublished). Because the ^{14}C isotope is associated with thymine, it is likely that these poorly retained species are low-molecular-weight oligonucleotide fragments or released thymine nucleotides. That they are present in urine within 1 h of dosing further implicates their early excision from oligonucleotide at a time when the chain-shortened oligonucleotides are apparent in circulation and tissue. These early eluting peaks were present at early time points but did not accumulate over time. These data indicate that there is both the formation and the clearance of low-molecular-weight metabolites of ISIS 2302 in laboratory animals.

Urinary and fecal excretion represent minor pathways for elimination of parent oligonucleotide. Assay methods specific to parent compound indicate that urinary excretion is dose-dependent and only accounts for less than 0.5% of the dose at clinically relevant doses (19,21,61). ISIS 5132 administered to mice over a dose range of 0.8–100 mg/kg resulted in increasing amounts of parent oligonucleotide apparent in urine (19). At the lowest dose, no measurable parent drug was seen while at the highest dose of 100 mg/kg, approximately 6% of the dose was excreted in the urine over a 24-h period immediately following injection. The majority of the oligonucleotide observed in urine was consistent with chain-shortened oligonucleotide metabolites. Even with this, the total oligonucleotide measured in urine following a dose of 4 mg/kg in mice was 3.7% of the administered dose.

Regardless of radioisotope used, fecal excretion of radiolabel has been consistently less than 5% of the administered dose (13,17,26). Some evidence of parent oligonucleotide excretion via the bile has been shown in our laboratories,

although the extent of excretion is very low (less than 1% of the dose; internal data, unpublished). Thus, urine and fecal excretion combined contribute little to the clearance of intact parent oligonucleotide. Thus reports of high levels of radioactivity excreted in urine and feces are related to shorter oligonucleotide metabolites and to label itself (not associated with oligonucleotide).

VI. SECOND-GENERATION PHOSPHOROTHIOATE OLIGONUCLEOTIDES

Several investigators have described favorable pharmacokinetic characteristics associated with 2′-ribose alkoxy modifications of phosphorothioate oligonucleotides (62–64). The 2′-ribose sugar modification essentially converts the deoxynucleotide to a ribonucleotide and, depending on the chemistry applied to the 2′ position, has the ability to provide profound protection from exonuclease metabolism (38,65,66). Because the backbone of this new generation of oligonucleotides remains as the negatively charged phosphorothioate, plasma-protein-binding characteristics are not substantially altered (internal data, unpublished). Therefore, excretion in urine is low (less than 5% of the administered dose) and, like their oligodeoxynucleotide cousins, distribution to tissue is broad and rapid, nearly complete within a few hours after intravenous administration. Distribution to tissues is similar to that of the PS oligodeoxynucleotides with liver, kidney, spleen, and lymph nodes exhibiting highest concentrations of oligonucleotide.

Although many of the pharmacokinetic attributes assigned to phosphorothioate oligodeoxynucleotides also apply to 2′-ribose-modified PS oligonucleotides, nuclease metabolism is markedly reduced. Circulating chain-shortened metabolites are not detected in plasma. Furthermore, CGE profiles of oligonucleotide distributed to tissue exhibit very few shortened metabolites even several weeks after a single injection of these compounds (Fig. 17). This robust characteristic has resulted in prolonged elimination half-lives in the body (67). Half-lives of intact 2′-methoxyethyl ribose oligonucleotides ranged from 7 to 15 days, depending upon tissue assayed, in monkeys, suggesting that once-weekly dosing may be feasible.

As previously described, the improved stability of these compounds appears to result in improved absorption following oral or intraintestinal instillation in rats (32,68) (Geary et al., manuscript in preparation, 2000). Since permeability in the intestine is low and absorption rate slow, retaining the intactness of the compound for several hours should result in improved fraction of dose absorbed. Estimates as high as 5% oral bioavailability of intact 20-mer 2′-modified oligonucleotide has been reported (Geary et al., 2000, manuscript in preparation).

Because the binding affinity of these second-generation chemistries to their target mRNA is also improved (69), potency both in vitro and in vivo is expected

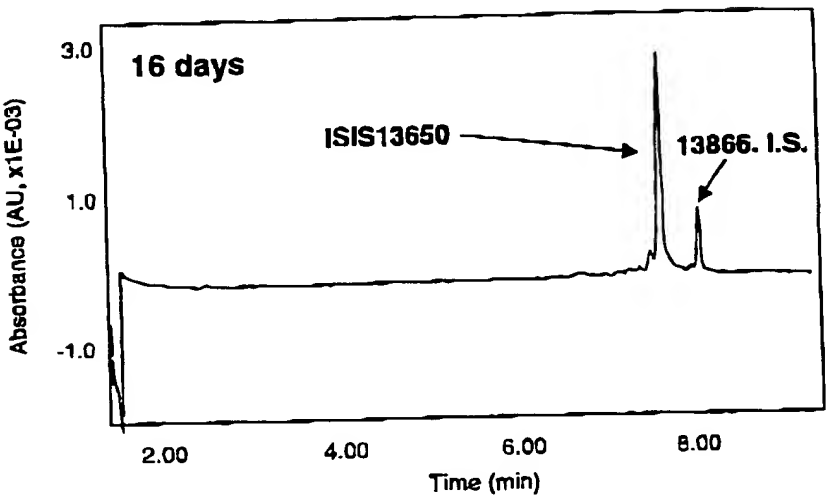

Figure 17 Representative electropherogram of extracted monkey kidney cortex collected 16 days after single dose administration of ISIS 13650, a 2′-methoxy ethyl-modified oligonucleotide targeting human c-*raf* mRNA. ISIS 13650 is modified on the six terminal nucleotides flanking both the 3′ and 5′ end of the 20-mer sequence.

to be improved compared to the first-generation PS ODN. The improved stability in vivo will further reduce the total doses required to elicit an antisense effect. Taken together, improved potency, bioavailability by nonparenteral routes of administration, and reduced dose schedule provide an attractive opportunity for treatment of unmet medical needs including chronic diseases such as inflammatory diseases, diabetes, and even cardiovascular diseases.

VII. PHARMACODYNAMICS CORRELATE WITH TISSUE PHARMACOKINETICS

The success of the antisense approach to therapy relies on the cellular distribution of the intact PS oligonucleotide. That is, antisense reduction of the expression of target mRNA relies upon suborgan distribution to the target cell type and, furthermore, subcellular distribution to the site of binding to its complement mRNA. The onset and duration of antisense therapeutic activity in the target cell type will provide the most reliable kinetic predictor for dose amount and frequency of administration.

The pharmacokinetics of PS oligonucleotides are characterized by rapid

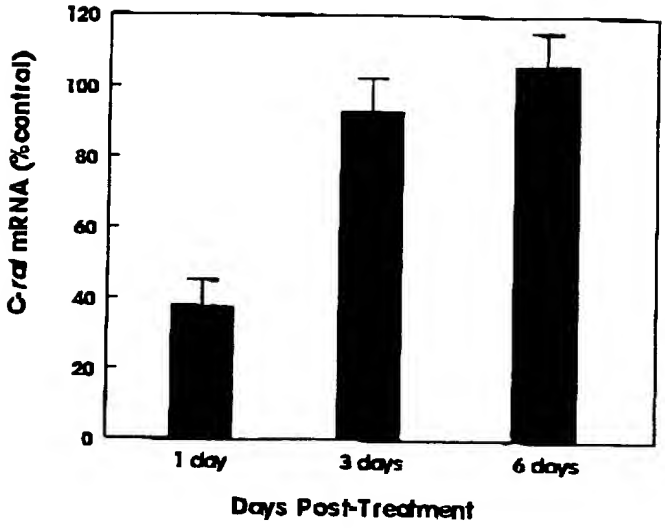

Figure 18 Northern analysis of c-*raf* kinase mRNA levels in whole-mouse liver measured as the percent of untreated control animals. By 3 days after antisense PS oligonucleotide treatment the mRNA levels had returned to baseline (100% of control). These pharmacodynamics are consistent with elimination kinetics of PS oligonucleotides from liver.

distribution from blood into tissues followed by prolonged residence in tissue. By 24 h after administration of a single dose of PS oligonucleotide, the oligonucleotide resides intracellularly in most tissues, excluding brain. Within tissues, the distribution of oligonucleotide varies by cell type but appears to be present in most cell types at some level (51). Intracellularly, more oligonucleotide appears to resides in the cytosol although higher concentrations are seen in the nucleus likely due to its smaller volume.

In preliminary in vivo pharmacodynamic experiments conducted in our laboratories, antisense pharmacodynamics in the liver closely parallel the pharmacokinetics of elimination of PS oligonucleotide from that organ (Fig. 18). The observed reduction in mRNA levels lasted approximately 1–2 days following intravenous administration of a mouse c-raf kinase antisense PS oligodeoxynucleotide while the half-life of elimination of PS ODN in the liver is approximately 2 days in mice. (Table 3). By 3 days after administration the mRNA had returned to baseline concentrations. Recently, investigators in our laboratories reported a single-dose pharmacology of ISIS 22023, a 2′-methoxy ethyl modified phosphorothioate oligonucleotide (second-generation) targeting mouse FAS mRNA. In this study, ISIS 22023 functionally down-regulated expression of the FAS mRNA in mouse liver for at least 10 days (70) following a single subcutane-

ous injection. By 14 days after a single dose of ISIS 22023 the mRNA levels had returned to baseline expression levels.

Thus, the pharmacokinetics and pharmacodynamics of PS oligonucleotides appear to be well correlated in vivo and support once-a-day or every-other-day systemic dosing for first-generation PS oligodeoxynucleotide chemistry. More convenient dosing schedules appear possible with once-weekly or once-every-other-week dosing of the second-generation 2′-ribose sugar–modified PS oligonucleotides indicated by initial evaluation of their pharmacokinetic and pharmacodynamic characteristics.

VIII. CONCLUSIONS

Pharmacokinetic studies of PS ODN demonstrate that they are well absorbed from parenteral sites, rapidly distributed broadly to all peripheral tissues, do not cross the blood-brain barrier, and are eliminated primarily by slow metabolism in tissues. Pharmacodynamics are well predicted by tissue uptake and elimination pharmacokinetics.

PS oligonucleotides are metabolized by exonuclease digestion. Further metabolism of the released mononucleotides follows normal endogenous metabolism of these compounds resulting in ultimate excretion of small-molecular-weight metabolites in urine and expired air. Excretion of the parent oligonucleotide in urine and feces is a minor pathway for elimination of these compounds with less than 1% of the dose excreted in the form of parent compound.

The pharmacokinetics of PS oligonucleotides in animals predict human pharmacokinetics and suggest similar distribution kinetics. In short, once-daily or alternating-day dosing should be feasible. This dosing schedule will result in continuous exposure of target tissues to the oligonucleotide and accumulation of oligonucleotide concentrations in tissues. Nevertheless, accumulation in plasma concentrations is not seen owing to rapid distribution into the tissue. This provides a favorable pharmacokinetic profile for this class of compounds. Improvements in the stability and ultimately in dosing routes for modifications of this chemical class (second-generation oligonucleotides) will provide an even greater breadth of use of antisense oligonucleotide therapy. Understanding the pharmacokinetics of PS oligonucleotides allows for rational improvements utilizing medicinal chemistry modifications. In general, the pharmacokinetic properties of this class of compounds appear to be largely driven by chemistry rather than sequence.

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